THE ROLE OF CYTOKINES IN BINGE-LIKE ETHANOL CONSUMPTION AND ETHANOL-INDUCED SEDATION

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology & Neuroscience (Behavioral Neuroscience).

Chapel Hill
2016

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ABSTRACT

John David Casachahua: The Role of Cytokines in Binge-Like Ethanol Consumption and Ethanol-induced Sedation

(Under the direction of Todd E.Thiele and Donald T. Lysle)

There is a growing body of research that is establishing a prominent role for the immune system in the brain. Recent studies have demonstrated a role for immune system messenger cytokines in modulating binge-like ethanol consumption. The goal of this dissertation is to examine the roles of proinflammatory cytokines, specifically IL-6, in binge-like ethanol consumption and ethanol-induced sedation. The experiments of Chapter 2 characterized the expression of IL-6 in the central amygdala, paraventricular nucleus of the hypothalamus (PVN), and other candidate regions of the brain in response to binge-like ethanol consumption through use of the "Drinking in the Dark" (DID) protocol followed by immunohistochemical procedures examining IL-6 immunoreactivity in the candidate regions of the brain. The most promising region of interest was the central amygdala (CEA), and in Chapter 3, experiments were performed with site-directed infusions of IL-6 receptor antagonist into the CEA to alter proinflammatory cytokine signaling and modulate binge-like ethanol consumption. These IL-6 receptor antagonists that were site-specifically administered on the test day on the third cycle of the DID protocol with ethanol reduced binge-like ethanol consumption. Following up on these findings were sucrose DID tests that were used to determine that consumption in general was not reduced by the application of this cytokine antagonist. Chapter 4 describes the experiments that

were run to assess whether IL-6 specifically modulated ethanol's sedative/ataxic properties, through the use of site-directed infusions of IL-6 receptor antagonist employed to alter proinflammatory cytokine signaling in the central amygdala. Mice were exposed to an intraperitoneal ethanol administration followed by the application of the IL-6 receptor antagonist. Subsequent to this protocol, mice were tested on their motor reflexes with a rotarod apparatus to determine the antagonist's impact on ethanol-induced sedation. Here it was established that IL-6 does not appear to modulate ethanol's sedative/ataxic properties. Together, these experiments indicate that IL-6 signaling critically modulates binge-like ethanol consumption after a history of binge-like ethanol consumption. The results suggest a potential therapeutic value for IL-6 antagonism in the reduction of binge ethanol drinking and a prophylactic approach against ethanol dependence.

ACKNOWLEDGEMENTS

My sincere thanks to:

The members of my dissertation committee: Todd Thiele, Donald Lysle, Regina Carelli, Kathryn Reissner, and Deborah Jones. Thank you for your thoughtful guidance and support throughout the stages of this dissertation.

My advisors, Todd and Don. Thank you for your solid advice throughout my graduate career. Your expertise and insights were invaluable towards the success of the dissertation. Also, Thank you for providing strong examples for me to aspire to in my future endeavors.

My lab colleagues: Thank you to the past and present members of the Thiele and Lysle labs. Your comments during lab meetings have helped me produce my best presentations and have helped me to view my research through a variety of different but rich perspectives. Also, thank you for your technical advice and assistance with the various experiments that made up this dissertation.

God and My parents: Thank you for support and guidance throughout my entire life. I would not be where I am without your care. I look forward to many more years of your love and friendship.

My siblings, and friends: Daniel, Amanda, and all my friends. Thanks for all the fun times! Let's continue to make new memories.

My previous mentors: Christian Reich and Michael Vigorito, Thanks for the advice and support that has provided me with a solid foundation for this neuroscience research career.

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α-MSH alpha-melanocyte stimulating hormone

ANOVA analysis of variance

BEC blood ethanol concentration

BLA basolateral amygdala

BNST bed nucleus of the stria terminalis

CEA central nucleus of the amygdala

CNS central nervous system

CRF corticotropin releasing factor

CRFR1 CRF receptor 1

DID drinking in the dark

ELISA enzyme-linked immunosorbent assay

g/kg gram per kilogram

GABA gamma-amino butyric acid

HMGB1 High mobility group box 1

HPA Hypothalamic–pituitary–adrenal

IACUC North Carolina Animal Care and Use Committee

ICV intracerebroventricular

IHC immunohistochemistry

IL-1 interleukin-1

IL-1RA interleukin-1 receptor antagonist

IL-6 interleukin-6

IL-10 interleukin-10

i.p. intraperitoneal

IR immunoreactivity

JAK Janus kinase

KO knock out

MAPK mitogen-activated protein kinase

MCP-1 monocyte chemoattractant protein-1

μg microgram

μg/μl microgram per microliter

μl microliter

mg milligram

mg/kg milligram per kilogram

mg/ml milligram per milliliter

ml milliliter

mRNA messenger RNA

NIAAA National Institute on Alcohol Abuse and Alcoholism

NIH National Institute of Health

NPY neuropeptide Y

PI3K phosphatidyl inositol-3

S.E.M. standard error of the mean

sIL-6R soluble interleukin-6 receptor

STAT signal transducer and activator of transcription

TLR4 toll-like receptor 4

TNF-α tumor necrosis factor alpha

VTA ventral tegmental area

w/v weight/volume

Chapter 1

General Introduction

Human binge ethanol drinking and the mouse model, "Drinking in the dark"

NIAAA (2004) defines binge drinking as a pattern of drinking leading to blood ethanol concentrations of greater than 0.08% or 80 mg/dl. This translates to be 4 to 5 drinks in 2 hours for the average adult. This pattern of drinking is most prevalent in both adolescent and adult populations. According to the Substance Abuse and Mental Health Services Administration's 2014 National Survey on Drug Use and Health, 24.7 % of adults aged 18 or older and 37.9% of college students aged 18-22 have engaged in binge drinking alcohol within the month of their survey. This binge drinking behavior increases the risk of accidental injury, increases mood disorders, increases aggressive and violent behavior, and impairs decision making and judgment (Gmel et al, 2006; Okoro et al., 2004; Shepherd et al., 2006; & Goudriaan et al., 2007). Heavy, prolonged binge drinking has been linked to long-term health consequences including heart disease, high blood pressure, and type 2 diabetes (Fan, Russell, Stranges, Dorn, & Trevisan, 2008). Furthermore, there is an increased risk for developing alcohol dependence in individuals that binge drink frequently (Courtney & Polich, 2009). Following the US Public Health Service guidelines, the estimated cost of excessive alcohol drinking in 2006 was $223.5 billion, with 76.4% or $170.7 billion in costs from binge drinking alone (Bouchery et al., 2011). A follow-up study published in 2015 reported that the estimated cost of excessive alcohol drinking in 2010 was $249 billion, with 76.7% or $191.1 billion in costs from binge drinking alone, which reflects

an escalating impact these abusive behaviors are having on this nation (Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015).

The "Drinking in the dark" (DID) procedure is an established mouse model of binge ethanol drinking developed by Rhodes and Colleagues (2005). For this procedure, 3 hours into the dark cycle mice have their water bottles replaced with 20% ethanol for 2 hours on days 1-3 for habituation, and for 4 hours on the day 4 Binge Test. Strong evidence that supports the face validity of this binge drinking animal model includes mice achieve blood ethanol concentrations (BECs) of 80 mg/dl or greater and most notably, these BEC levels often exceed the NIAAA operational definition of a binge for humans, which is 80 ml/dl ≈0.08% BEC. Also, mice exhibit behavioral intoxication as measured by deficits in motor behavior in rotarod and balance beam tests (Rhodes et al., 2007). Moreover, excessive ethanol drinking associated with DID procedures does not appear to be driven by caloric need (Lyons et al., 2008).

The neuroimmune system

In order to understand the potential roles that cytokines might play in ethanol consumption and sedation, it is important to have a basic understanding on how the neuroimmune system works and what functions the cytokines have when ethanol isn't in the body. Innate immunity is the immune system's first line of defense against invading organisms or pathogens coming into the body. Adaptive immunity, by contrast, involves the synthesis of antibodies to deal with specific viruses or other threats that escape the innate immune system's defenses. Unless the body was previously exposed to these pathogens with subsequent antibodies circulating, the adaptive immune response may take a while to respond effectively to bodily threats. Thus, the innate immune system is always active while the adaptive system is usually silent unless activated (Sompayrac, 2008). Because of the innate immune system's ever-present

activity, these immune components are found to be the most relevant focus for studies in communications or signaling between the nervous and the immune (or neuroimmune) systems. Macrophages are the immune system's sentinels which have the job of monitoring the body for threats to the neuroimmune system. These macrophages have special pattern recognition receptors called toll-like receptors that recognize specific types of immune threats. Microglia are specialized macrophages that operate in the central nervous system. These microglia or glial astrocyte cells generate messenger molecules called cytokines to communicate with other cells. Some of the cytokines generated include interleukin-1 (IL-1), IL-6, IL-10, and Tumor necrosis factor –alpha (TNF-α). Notably, IL-1 expression can trigger IL-6 and TNF-α expression, and the over-expression of these cytokines can lead to the anti-inflammatory cytokine, IL-10, which acts to regulate these other cytokines (Sompayrac, 2008; Shastri, Bonifati, & Kishore, 2013).

Generally, these immune processes can have either protective or pathogenic effects depending on the circumstances and duration of their expression. Indeed, if stress or a biological infection is great enough, neurons can recruit or generate immune elements to respond to these challenges. In fact, some immune components can also contain receptors for neurotransmitters (e.g., norepinephrine, epinephrine, & acetylcholine) and neuropeptides (e.g., neuropeptide Y [NPY], corticotropin releasing factor [CRF], & α-melanocyte-stimulating hormone [α-MSH]). In fact, NPY's actions on macrophages have been found to reduce IL-6 expression. Depending on these biological circumstances, immune elements can either be neuroprotective or can destroy dysfunctional/biologically compromised neurons. (Walsh, Muruve, & Power, 2014; Sternberg, 2006).

The central nervous system can react to homeostatic challenges through local, regional, or systemic actions which may also include hormonal routes. The hypothalamic-pituitary-adrenal

(HPA) axis is a group of glands or structures that work together to regulate stress, neuroendocrine, and digestion processes but can also mediate and interact with innate immune components (Sternberg, 2006). The Vagus nerve is a nerve that acts as a communication relay between the heart, digestive tract, and the medulla oblongata in the brain. This bidirectional nerve can relay several types of signals such as hunger or satiety, but can also relay immune signals between the central nervous and the peripheral immune system (Rosas-Ballina et al, 2015; Sternberg, 2006).

The blood-brain-barrier is a tight junction (400 dalton or less permeability) of endothelial cells that regulate communication between the central nervous system (CNS) and the peripheral immune system. This barrier may allow some cytokines to signal, or be transported through these structures, but it generally protects the CNS from pathogens that the peripheral immune system might be fighting against (Banks, 2015). However, Alfonso-Loeches and others (2015) have reported that chronic exposure to ethanol may compromise the ability of the blood-brain-barrier to protect the brain from neuroinflammation, as demonstrated by greater expression of inflammatory genes in the cultured microglia of ethanol treated mice. This compromised barrier may be due to peripheral liver inflammation. The liver acts as a barricade between the gut and the rest of the body, and when there is significant inflammation, resident immune cells such as Kupffer cells generate or influence an immune response which can include cytokines and monocytes that can potentially force pass this barrier (D'Mello, & Swain, 2014; Rubio-Araiz et al., 2016).

Additionally, there are small brain regions that do that have blood-brain-barrier protection and thus greater permeability which are classified as circumventricular organs. Several of these organs have connections to the hypothalamus in the brain (Banks, 2016). The

hypothalamus is one brain region that can generate a strong central cytokine response to peripheral immune challenges. In fact, IL-6 is the primary cytokine responsible for modulating inflammation and fever symptoms (Murta, Farias, Pitossi, & Ferrari, 2015). The amygdala is another brain region that has a prominent central immune presence. The amygdala is considered the hub or coordinator for receiving and integrating peripheral immune signals (Engler et al., 2011.) Finally, Louveau and colleagues (2015) recently discovered lymphatic vessels that have been found in the central nervous system which allow transport of fluids and immune cells from the cerebrospinal fluid and the lymph nodes into the brain. However, the implications of this new discovery on a potential neuroimmune communication pathway have yet to be determined.

The critical roles of interleukin 6 in the central nervous system

Interleukin-6 plays a variety of roles in the body such as regulating inflammation, contributing to neurogenesis, as well as endocrine roles. This cytokine may be produced by neurons, microglia, astrocytes, or endothelial cells. IL-6 (22-28 kilodalton) may bind to a membrane bound receptor (IL-6R, gp80, 80 kilodalton α-type receptor) or to a soluble receptor (sIL-6Rα). IL-6R is expressed in limited amounts, while sIL-6R is ubiquitous or pervasive. Regardless of which receptor IL-6 binds to, IL-6 needs the ubiquitous protein gp130 (130 kilodalton β-type receptor) for signaling (Erta, Quintana, & Hidalgo, 2012; Hunter, & Jones, 2015). The IL-6 receptor is synthesized in the endoplasmic reticulum and will appear 45 minutes after synthesis at the cell surface. Notably, the half-life of IL-6R and gp130 is approximately 2-3 hours. However, an increased presence of IL-6 will not escalate the rate of IL-6R or gp130 degradation due to the diverse cell type mechanisms that process IL-6 (Gerhartz et al, 1994).

IL-6 is a critical cytokine that controls the transition from innate to adaptive immune processing. However, the roles of IL-6 may be heavily context dependent (Erta, Quintana, &

Hidalgo, 2012). IL-6's role may depend on the level of inflammation in the affected regions. IL-6, in concert with sIL-6Rα has been found to modulate the transition between acute and chronic inflammation. IL-6 has been suggested to be protective in low levels, but to be proinflammatory during chronic inflammation. One way that IL-6 has been found to be protective is by inducing production of the anti-inflammatory interleukin-1 receptor antagonist (Gabay, 2006). It would seem by most accounts that IL-6's main goal is to maintain homeostasis in the body. Yet, how might one understand how IL-6 might be able to potentially have different seemingly contradictory roles in the body?

Interleukin-6 has three signaling pathways that it is known to act on within the body. The least relevant pathway to the topic of neuroimmune signaling is the role that IL-6 plays in the skeletal muscle system. In this system, IL-6 is produced as a myokine which is essentially a cytokine secreted by muscles. In this capacity, IL-6's primarily role appears to be to reduce inflammation and enhance functionality within the muscles, as well as enter the blood stream and impact the body's metabolism. This, in turn, can have an impact on body mass and is presently investigated in obesity research. As a myokine, IL-6 follows a different pathway of signaling and expression than as the cytokine IL-6 (Pal, Febbraio, & Whitham, 2014; Guijarro, Laviano, & Meguid, 2006).

The two most relevant pathways that IL-6 acts on within the neuroimmune system are the classic signaling and the trans-signaling pathways. Classical signaling is when IL-6 binds to IL-6R and gp130. Classical signaling helps maintain homeostasis and can be neuroprotective. Trans-signaling is when IL-6 binds with sIL-6R and gp130. Trans-signaling is largely responsible for chronic inflammation (and depression) since the pervasiveness of these binding elements allow IL-6 to affect cells that do not express IL-6R. Trans-signaling has been suggested

to be involved in gut permeability, which is one of the proposed ways that binge ethanol exposure has been proposed to cause cytokines to circulate and impact the neuroimmune system. Soluble gp130 is one of the endogenous antagonists to this trans-signaling. This antagonist works by competing with the gp130 protein for binding to the IL-6 complex (Maes, Anderson, Kubera, & Berk, 2014; Jostock et al., 2001).

In neurons, IL-6 can act as a neuromodulator. For example, Hernandez and colleagues (2016) recently reported that transgenic mice that were engineered to generate increased astrocytic induced IL-6 expression demonstrated altered synaptic function from acute ethanol exposure. IL-6 has been found in the PVN and other regions of the hypothalamus, the hippocampus, and also the cerebellum. Within these structures, the majority of IL-6 mRNA expression was found in neurons (Benrick, et al., 2009; Sallmann, et al., 2000; Aniszewska et al., 2015; Jankord et al., 2010). IL-6 has been detected in both cholinergic and GABAergic neurons. IL-6 is considered a neuropoietic cytokine because it promotes neuronal survival. Depending on the concentrations of IL-6, this cytokine can protect against NMDA excitotoxicity, which is a factor in chronic alcohol drinking. IL-6 may influence neurons directly, but may also act as a messenger between glia and neurons (Juttler, Tarabin, & Schwaninger, 2002). In fact, some preliminary research suggests that IL-6 may act pre-synaptically or post-synaptically to alter neurotransmitter release (Gruol, 2015; Crowley, Cryan, Downer, & O'Leary, 2016). Yet, this aforementioned type of neuronal meditated IL-6 signaling would most likely be considered classical signaling since inflammation does not appear to be the endpoint.

The different roles of cytokines in ethanol consumption

There are several cytokines that have been implicated in ethanol consumption behaviors such as IL-1, IL-6, IL-10, TNF-α, and MCP-1. These cytokines have different roles in the brain

based upon the levels of ethanol exposure. Alcohol exposures have been found to act on the toll-like receptors TLR4 and IL-1R (Fernandez-Lizarbe, Pascual, Gascon, Blanco, & Guerri, 2008). Toll-like receptors then produce cytokines. IL-1 has been found to induce IL-6 and TNF-α. The anti-inflammatory cytokine IL-10 is later synthesized to counter the proinflammatory cytokines IL-1, IL-6 and TNF-α. Ethanol has been linked to the production of TNF-α, MCP-1, IL-6, and IL-1, and the use of cytokine neutralizing antibodies blunt cytokine expression and attenuates ethanol sensitization to glutamate neurotoxicity (Emanuele et al., 2005; Zou, & Crews, 2010).

Pro-inflammatory interleukin 1(β) has been demonstrated to have an increased expression within the neurons and astrocytes of the hippocampi of postmortem alcoholic brains (Zou & Crews, 2012). Genetic polymorphisms of IL-1β have been found with several alcohol dependent patients, in comparison with non-dependent healthy controls (Liu, Hutchinson, White, Somogyi, & Coller, 2009). Genomic microarrays have also identified IL-6 signaling as a contributor gene to alcohol preference (Mulligan et al., 2006). Kane and colleagues (2014) report ethanol-induced increases of pro-inflammatory IL-6 mRNA in the cerebellum of C57BL/6J mice in response to oral gavage of 6 g/kg ethanol. Also, within the same study pro-inflammatory MCP-1 mRNA levels were increased within the hippocampus, cerebellum, and cerebral cortices of these mice. In support of the ethanol-induced IL-6 changes, two other studies found ethanol applied to astroglia or microglia in culture caused an increase in IL-6 expression (Boyadjieva, & Sarkar, 2010; Sarc, Wraber, & Lipnik-Stangelj, 2010). In another postmortem study of alcoholic brains, high concentrations of MCP-1 were found in the VTA and the amygdala (He & Crews, 2008).

Marshall and colleagues (2013) exposed rats to a binge ethanol paradigm, and discovered increased expression of anti-inflammatory IL-10 seven days after the ethanol exposure. Qin and colleagues (2008) report increased pro-inflammatory TNF-α expression in

whole brain assessments of C57BL/6J mice after being exposed to oral gavage of 5 g/kg ethanol. Also, Emanuele and colleagues (2005) exposed rats to a chronic ethanol paradigm which increased TNF-α and IL-6 expression in their hypothalamus(i). Additionally, alcohol dependent patients admitted to a hospital show the highest TNF-α serum levels and liver dysfunction/disease as compared to moderate, light, and abstaining alcohol drinkers. Furthermore, the TNF gene polymorphism of the -238A allele has been associated with a high prevalence for liver disease (Gonzalez et al., 2008). In fact, the liver contains Kupffer cells which synthesize TNF-α and other cytokines. TNF-α passed into serum can induce MCP-1 and TNF-α in the brain, such that TNF-α can remain in the brain for at least 10 months and cause neurodegeneration (Crews et al., 2006). TNF-α is a particularly dangerous cytokine that has been associated with neuronal death, specifically in dopamine cells (Shastri, Bonifati, & Kishore, 2013). Thus, it could be argued that IL-1 induces other cytokines, (e.g. IL-6), with TNF-α and MCP-1 being some of the cytokines present before neuronal death.

However, one of the strongest examples of the role of central immune gene expression in the *modulation* of ethanol consumption is found in a study by Liu and colleagues (2011). In this study, the research group utilized RNA silencing technologies (siRNA) to selectively knock out TLR4 in the central amygdala of alcohol preferring rats. This TLR4 knockdown reduced operant responding for ethanol, but not responding for the more palatable sucrose. Thus, within this study the central immune response was altered or blocked, which in turn reduced alcohol consumption. Other examples include mice with the Knock-Out (KO) of IL-1 and IL-6 genes showing reduced ethanol consumption within two bottle alcohol preference tests (Blednov et al., 2012).

The cytokine contribution to alcohol dependence: A potential model of cytokine actions

In consideration of the cytokine roles in the brain in response to normal and/or ethanol modulated processes, there is the implication that pro-inflammatory cytokine expression increases binge-like ethanol drinking. One model that has been postulated to account for the actions of the central cytokines within the pathway to dependence or addiction is the Allostasis model developed by Dr. George Koob. Homeostasis is characterized by the bodily processes that work to maintain the functionality and survival of an organism. Allostasis refers to the process where the same adaptive processes that work within homeostasis become dysregulated and these adaptive processes change to attain stability, yet these changes push the regulatory systems outside the normal set-point into a potentially pathological set-point (Koob, 2003; Koob & Le Moal, 2001). Potentially the impairment in the homeostatic role of IL-6 might be responsible for the inability of the homeostatic mechanisms to attain the original normal set-points.

The descent into alcohol addiction is characterized by experiences of positive and negative reinforcement. At first, alcohol activates the brain reward systems and generates a pleasurable experience. This period of positive reinforcement causes the alcohol user to binge drink alcohol in pursuit of the initial pleasures of alcohol use. Yet, as the body adapts to the continued binge exposures of alcohol, the body does not respond in the same ways to this drug. After several binge exposures to alcohol, cessation of alcohol drinking (abstinence) causes the aversive withdrawal state. In this period of negative reinforcement, alcohol is then taken to reduce the aversive effects of the withdrawal experience (i.e., relapse). Binge cycles of alcohol use and withdrawal alter the normal set-points into an allostatic state. While in the allostatic cycle, prior set-points are no longer attainable. Thus, the perpetual cycles eventually descend into a pathological state (Koob & Le Moal, 2001; Koob, 2003). These cycles are easily represented

by multiple binge-like cycles of DID in mice. Mice are expected to demonstrate similar behaviors to humans in response to this extended binge-like alcohol exposure.

The reward circuitry involved with alcohol reinforcement includes the extended amygdala (including the BNST), the lateral hypothalamus, the nucleus accumbens, and the ventral tegmentum area (VTA) (Koob, 2003). These same brain areas (with the exception of the VTA) have been found to host interactions between the neurotransmitters (serotonin and dopamine) implicated in the alcohol allostasis model and the cytokines IL-1(β), IL-6, and TNF-α (Brebner, Hayley, Zacharko, Merali, & Anisman, 2000). Additionally, the central amygdala was found to host interactions between TNF-α and GABA (Knapp et al, 2011). Furthermore, the innate immune response to pathogens has been found to have interactions with the opioids, glucocorticoids, NPY, and CRF throughout the central nervous system (Sternberg, 2006). Thus, it could be argued that cytokines (such as the homeostatic IL-6) interact with key neurotransmitters and neuropeptides in regions implicated in alcohol reinforcement processes. While the framework for the allostatic model is not complete, given the cited evidence, it seems likely that cytokines contribute vitally to alcohol dependence/pathology.

Potential role of the pro-inflammatory cytokines in the relation between sensitivity to ethanol-induced sedation and ethanol intake

Work by Dr. Mark Shuckit (1994) has demonstrated that a low level of response or sensitivity to ethanol, as evident by delayed intoxication or sedation, has been associated with high risk for later alcoholism. Furthermore, children of alcoholics will also show a higher risk for alcoholism, when compared to children from a non-alcoholic lineage. Shuckit and colleagues (2011) suggest that the low sensitivity to ethanol may be seen as both a genetic and environmental contributor to alcoholism. The genetic component lies in the fact that

approximately 50% of low alcohol response is genetically mediated. The environmental component is due to the higher number of drinks needed to achieve the desired ethanol effects. Paralleling these facts, cytokines have been demonstrated to produce an environmental factor in ethanol drinking, (as previously stated), and cytokine gene polymorphisms have been implicated with higher incidences of alcohol dependence (Liu et al., 2009; Gonzalez et al., 2008). Thus, pro-inflammatory cytokines may play a role in ethanol sensitivity.

Ethanol drinking has been shown to increase pro-inflammatory cytokine expression, and cytokine expression increases ethanol drinking. Based upon a review of the literature, it would seem that pro-inflammatory cytokines have an antagonistic relation with ethanol sensitivity/sedation. Thus, pro-inflammatory cytokines would be expected to lower sensitivity to sedation, and would increase ethanol consumption. Sedation may be motivated by an energy metabolism factor, and may actually work to stop a potential cytokine induced illness. Indeed, cytokine antagonists may increase ethanol-induced sedation.

However, it is also possible the cytokines may generate a high sensitivity/sedation due to a potential over-response of cytokines which would then usher in sickness or sedation to cope with the cytokine response. In this scenario, cytokine antagonists would work to normalize the cytokine over-reaction. Wu and colleagues (2011) report that the *high peripheral dose* of 100 mg/kg/i.p. IL-1RA reduced ethanol-induced sedation (sleep time) and motor impairment. Additionally, this research team (2011) also reports that TLR4 KOs and Myd88 KOs (immune gene knock-out mice) show reduced sedation and motor impairment than matched controls. Corrigan and colleagues (2014) also report that TLR2 KOs show minimal sedation behaviors in comparison to wild-type controls. Yet, high doses of antagonist or gene deletion may not present that best tools for assessing IL-1's actions. Gene knockdown methods are preferable tools to

avoid developmental or immune system defects, or compensations from never possessing the gene (KO mice). Also, gene knockdown methods allow temporal and spatial characterization or manipulation of these genes that is not easily achieved with KO mice or peripheral injections. In contrast to the previously referenced studies, Vicente-Rodriguez and colleagues (2014) report that mutant mice that overexpress the cytokine pleiotrophin show enhanced ethanol preference and reduced sedation/ataxia in response to ethanol administration. As evident by these aforementioned studies, prior research has provided mixed results concerning the role of cytokines in ethanol sensitivity and sedation. However, the anticipated result for these studies, based upon prominent alcoholism theories, is for pro-inflammatory cytokines to lower sensitivity to sedation, and increase ethanol consumption.

Goals of Current Dissertation

The overarching goal of this dissertation is to examine the roles of proinflammatory cytokines, specifically IL-6, in binge-like ethanol consumption and ethanol-induced sedation. The experiments of chapter 2 characterize the expression of IL-6 in the central amygdala, paraventricular nucleus of the hypothalamus (PVN), and other candidate regions of the brain in response to binge-like ethanol consumption. Within these experiments, mice were exposed to 1 or 3 cycles of binge-like drinking cycles through use of the "Drinking in the Dark" (DID) protocol consisting of either 20% v/v ethanol or water. Following the DID protocol, mice brains were analyzed through immunohistochemical procedures examining IL-6 immunoreactivity in candidate regions of the brain implicated in the neurobiological responses to ethanol. In chapter 3, experiments were performed with site-directed infusions of IL-6 receptor antagonist to alter proinflammatory cytokine signaling and determine the critical regions in which IL-6 modulates binge-like ethanol consumption. These IL-6 receptor antagonists were site-specifically

administered on the test day of the DID protocol with ethanol. Following up on these findings were sucrose DID tests that were used to determine that consumption in general was not reduced, or that taste perception was not altered by the application of this cytokine antagonist. Chapter 4 describes the experiments that were run to assess whether IL-6 specifically modulated ethanol's sedative/ataxic properties, through the use of site-directed infusions of IL-6 receptor antagonist employed to alter proinflammatory cytokine signaling in the central amygdala. Mice were exposed to an intraperitoneal ethanol administration followed by the application of the IL-6 receptor antagonist. Subsequent to this protocol, mice were tested on their motor reflexes with a rotarod apparatus to determine the antagonist's impact on ethanol-induced sedation.

Chapter 2

Binge-like ethanol consumption effects on central interleukin-6 expression

Introduction

A growing body of evidence suggests that central cytokines play critical roles of modulating neurobiological responses to ethanol. IL-6 is one of the noteworthy cytokines that have been previously implicated in alcohol consumption. For example, ethanol has been linked to the production of the cytokines: TNF-α, MCP-1, IL-6, and IL-1 (Emanuele et al., 2005; Zou, & Crews, 2010). Kane and colleagues (2014) report ethanol-induced increases of pro-inflammatory IL-6 mRNA in the cerebellum of C57BL/6J mice in response to oral gavage of 6 g/kg ethanol. Emanuele and colleagues (2005) exposed rats to a chronic ethanol paradigm which increased TNF-α and IL-6 expression in their hypothalamus. In support of the ethanol induced IL-6 changes, two other studies found ethanol applied to astroglia or microglia in culture caused an increase in IL-6 expression (Boyadjieva, & Sarkar, 2010; Sarc, Wraber, & Lipnik-Stangelj, 2010). Genomic microarrays identified IL-6 as a contributor gene to alcohol preference (Mulligan et al., 2006). Finally, deletion of IL-1 and IL-6 genes resulted in reduced ethanol consumption within 2-bottle preference tests (Blednov et al, 2011).

The present study examined the role of brain IL-6 in excessive binge-like ethanol drinking in C57BL/6J mice, by assessing IL-6 immunoreactivity in key brain regions previously implicated in modulating neurobiological responses to ethanol. Mice were exposed to a 4 day "drinking in the dark" (DID) binge-like ethanol consumption procedure, which promotes high

ethanol intakes with associate blood ethanol concentrations in excess of 80 mg/dl (Sparta et al., 2008; Lowery et al., 2010).

Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were approximately 6-8 weeks old and weighed between 20-25g at the beginning of experimental procedures. Mice were individually housed in polypropylene cages with corncob bedding and ad libitum access to standard rodent chow (Purina RMH 3000, Tekland, Madison, WI) and water, except where noted in experimental procedures. The colony rooms were maintained at 22ºC with a reverse 12-hr/12-hr light/dark cycle with lights out at 10 a.m. Mice were run in two cohorts for this study. Cohort 1 consisted of 30 mice used in water and sucrose intake experiments. Cohort 2 consisted of 30 mice used in water and ethanol intake experiments. All experimental procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC) and complied with the NIH Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

Binge-Like Drinking Procedures

The "drinking in the dark" (DID) protocol involves a 4-day procedure and is an animal model of binge ethanol consumption (Rhodes et al., 2005). Throughout the present experiments, mice remain in their home-cages in the vivarium. Beginning 3 hours into the dark cycle, water bottles were removed from the home-cage and replaced with a bottle containing a solution of 20% (v/v) ethanol. On days 1-3, ethanol bottles remained on the cages for 2 hours before removal and replacement with water bottles. On day 4 (the test day), procedures were the same as on days 1-3 except mice had access to ethanol for 4 hours. Mice were exposed to 1 or 3 cycles

(weeks) of the DID protocol with ethanol, or 3% sucrose in a separate experiment. Control groups underwent 3 cycles of the DID protocol with water. Mice were euthanized via ketamine (10mg/kg) /xylazine (100mg/kg) overdose and transcardially perfused with 0.1M phosphate buffered saline followed by 4% paraformaldehyde at the end of the DID period. Brains were extracted and sectioned using a vibratome. Tail blood samples (10µl) were collected after Day 4 Binge Test, though blood ethanol concentrations (BECs) could not be calculated due to equipment failure. Ethanol consumption is expressed as g/kg. Sucrose consumption is expressed as ml/kg.

Immunohistochemistry Procedures

Free floating sections were rinsed in phosphate buffered saline (PBS) and endogenous peroxidases quenched with 0.6% H_2O_2 in PBS. Following additional washes and a blocking step (PBS/ 0.1% of triton-X / 3% goat serum; Vector Laboratories, Burlingame, CA), sections were incubated in rabbit IL-6 (1:2000, Abbiotec, San Diego, CA) primary antibody for 72 hours at 4˚C. Primary antibody was washed away using the blocking solution, and sections were then incubated in biotinylated goat anti-rabbit secondary antibody and detected with avidin-biotin-peroxidase complex (ABC elite kit, Vector Labs) with the chromagen, 3,3'-diaminobenzidine tetrahydrochloride (Polysciences; Warrington, PA). Sections were mounted and coverslipped with SHUR/Mount™ (Triangle Biomedical Sciences; Durham, NC). Images were taken of the entire slide at 100x magnification with a Zeiss Axio Zoom V16 microscope (Zeiss; Jena, Germany). Slides were coded to ensure experimenter blindness to treatment conditions for quantification. Subregions of the amygdala or the hypothalamus were traced separately for each slide. IL-6 immunoreactivity was determined using ZenPro 2012. Immunopositive pixels were

determined by optical density with an experimenter determined threshold. Data points are expressed as percent area, which is pixels/area in micro meters.

Data Analysis

One-way and two-way analyses of variance (ANOVAs) were used to assess group differences in IL-6 IR as a function of binge-like ethanol consumption versus water intake or of binge-like sucrose consumption versus water intake. LSD post-hoc tests were used to determine specific differences in IR between the continuous water and all other groups. Significance was accepted at $p<0.05$; all data is presented as mean ± SEM. For IR data, some animals were excluded from analysis (due to brain region availability or outlier IR staining of greater than two standard deviations from the mean as determined by the "Grubbs test for outliers"), which accounts for differences in degrees of freedom between similar analyses.

Results

No significant differences in binge-like ethanol consumption or sucrose consumption

Mice in the 1 week or 3 week DID procedure consumed similar amounts of 20% ethanol on day 4 during the Binge Test. On day 4, mice exposed to 1 or 3 weeks of the DID procedure consumed (5.22±0.24, 5.52±0.67g/kg, respectively; $F(1,17)= 0.420$, $p=0.692$), (**Figure 2.1A**). This level of consumption is associated with BECs of ~100mg/dl (Lowery-Gionta et al., 2012). Mice in the 1 week or 3 week DID procedure with 3% sucrose consumed similar amounts of sucrose (176.70±13.68, 188.1±10.68 ml/kg, respectively; $F(1,9)= 0.403$, $p=0.541$). Also, as would be expected, mice who had access to water only consumed significantly less fluid than the sucrose exposed mice (52.52±4.23, $F(2,13)=45.58$, $p=0.0001$). Fluid consumption (ml/kg) during a separate 1 or 3 week DID procedure with 3% sucrose or water (**Figure 2.1B**)

Multiple cycles of DID with ethanol are associated with increased IL-6 expression in the central amygdala (CEA)

Mice were exposed to 1 or 3 weeks of DID with 20% ethanol, or 3 weeks of monitored water consumption within the primary IR experiment. In a separate experiment, mice were exposed to 3 weeks of DID with 3% sucrose, or with 3 weeks of monitored water consumption. Mice exposed to DID with ethanol demonstrated significantly greater IL-6 IR in the CEA than mice exposed to 3 weeks of water ($F(2,24)=3.481$, $p=.047$). This effect was primarily driven by mice exposed to 3 weeks of DID with 20% ethanol ($p=.014$) and not mice exposed to 1 week of DID ($p=.121$), versus water controls (**Figure 2.2A**). Notably, there were no significant differences between the 1 or 3 week DID ethanol groups and water controls with IL-6 IR in the BLA ($F(2,24)= .939$, $p=.405$) (**Figure 2.2B**). In the separate experiment, mice exposed to 3 weeks of DID with sucrose demonstrated no significant differences with IL-6 IR in the CEA than mice exposed to 3 weeks of water ($F(1,6)=0.024$, $p=.882$). Grubbs test for outliers found two outliers that were subsequently removed from data analysis: One mouse had a score of 25.05 in the sucrose group, and another mouse had a score of 29.86 in the water group. (**Figure 2.2C**). All data points are expressed as percent area, which is pixels/area in micro meters. Representative photomicrographs of IL-6 IR in the BLA and CEA are shown at 100X (**Figure 2.3**).

Multiple cycles of DID with ethanol are associated with increased IL-6 expression in the paraventricular nucleus of the hypothalamus (PVN).

Mice were exposed to 1 or 3 weeks of DID with 20% ethanol, or 3 weeks of monitored water consumption within the primary IR experiment. In a separate experiment, mice were exposed to 1 or 3 weeks of DID with 3% sucrose, or with 3 weeks of monitored water consumption. Mice exposed to DID with ethanol demonstrated significantly greater IL-6 IR in the PVN than mice exposed to 3 weeks of water ($F(2,23)=4.237$, $p=.027$).This effect was

primarily driven by mice exposed to 3 weeks of DID with 20% ethanol (p=.008) and not mice exposed to 1 week of DID (p=.258), versus water controls (**Figure 2.4A**). In the separate experiment, mice exposed to 1 or 3 weeks of DID with sucrose showed no significant differences in IL-6 IR in the PVN than mice exposed to 3 weeks of water ($F(2,12)=2.006$, p=.177), suggesting an ethanol specific effect on IL-6 expression at both 1 and 3 weeks of DID exposure (**Figure 2.4B**). Additionally, there were no significant differences between the 1 or 3 week DID ethanol groups and water controls with IL-6 IR in adjacent regions of the hypothalamus, such as the arcuate nucleus ($F(2,21)=1.504$, p=.245). All data points are expressed as percent area, which is pixels/area in micro meters. Representative photomicrographs of IL-6 IR in the PVN are shown at 100X (**Figure 2.5**)

Multiple cycles of DID with ethanol did not alter IL-6 expression in the lateral septum, nucleus accumbens, or bed nucleus of the stria terminalis (BNST).

Mice were exposed to 1 or 3 weeks of DID with 20% ethanol, or 3 weeks of monitored water consumption within the primary IR experiment. There were no significant differences between the 1 or 3 week DID ethanol groups and water controls with IL-6 IR in the dorsal, intermediate, or ventral lateral septum ($F(2,24)= .450$, p=.643; $F(2,25)=.586$, p=.564; $F(2,25)= .115$, p=.892; respectively). There were no significant differences between the 1 or 3 week DID ethanol groups and water controls with IL-6 IR in the nucleus accumbens core or shell ($F(2,23)=.343$, p=.713; $F(2,23)=1.587$, p=.226; respectively). There were no significant differences between the 1 or 3 week DID ethanol groups and water controls with IL-6 IR in the BNST ($F(2,24)= .010$, p=.990). These regions were chosen based upon the roles that they play with reward processes, and these non-significant results suggest an ethanol consumption mediated circuit involving IL-6 in both the CEA and the PVN, without the contributions of the lateral septum, nucleus accumbens, or BNST.

Discussion

Relative to water drinking controls, IL-6 immunoreactivity (IR) is up-regulated in the central amygdala and the paraventricular nucleus of the (PVN) hypothalamus of mice with a history of three binge-like ethanol drinking cycles. This effect is not found in mice that were only exposed to one binge-like ethanol drinking cycle. In a separate study comparing sucrose versus water drinking induced IL-6 IR, no significant differences with IL-6 IR were found in the central amygdala or the PVN. This sucrose consumption finding suggests that IL-6 has unique actions in response to ethanol consumption, which are not found with consumption of other salient reinforcers. Additionally, IL-6 IR is unaffected in the (adjacently located) basolateral amygdala of mice with a history of three binge-like ethanol drinking cycles. Notably, IL-6 IR is not increased in the BNST or the nucleus accumbens in response to one or three cycles of binge-like ethanol drinking. This seems to indicate that IL-6 does not have a direct effect on the regions of the reward circuitry indicated in Koob's (2003) allostatic model of alcohol addiction. Also, IL-6 IR is not increased in the lateral septum in response to one or three cycles of binge-like ethanol drinking. The lateral septum is a non-classical reward related region of interest that has been regarded as important for ethanol consumption as described by Ryabinin, Bachtell, and colleagues (2003, 2003, & 2008). Additionally, Breese and colleagues (1984) described the septum as a region that is important for mediating ethanol induced motor impairment or sedative behaviors. However, the significant effects in the central amygdala and the PVN suggest that increased IL-6 expression in the brain is site-specific in response to alcohol consumption. The fact that three cycles or weeks of ethanol exposure, and not one week of ethanol exposure, are needed to produce the enhanced IL-6 expression over the water controls suggests that IL-6 actions are more relevant in subjects with an extended history of binge-like ethanol consumption.

While the BECs were not able to be analyzed due to equipment failure, the high level of ethanol consumption found with these experimental animals is associated with BECs of ~100 mg/dl found in previous studies (Lowery et al., 2012).

The fact that enhanced IL-6 expression was found in the PVN and the CEA is especially significant since the importance of these structures has been previously demonstrated in prior ethanol consumption studies. For example, neuropeptide Y signaling in the PVN and the CEA has been found to critically modulate ethanol consumption (Sparrow et al., 2012; Kelley et al., 2001). Also, the neuropeptide corticotrophin releasing factor (CRF) which has been found to be greatly expressed in the PVN and the CEA, has also been found to critically modulate ethanol consumption (Wills, Knapp, Overstreet, & Breese, 2010).

Prior immune studies offer a different view of the role of IL-6 in the CEA and the PVN. The central amygdala is commonly viewed as one the brain regions responsible for processing peripheral immune signals to the brain. In fact, Engler and colleagues (2011) reported that an intraperitoneal injection of bacterial lipopolysaccharide (LPS) induced pronounced IL-6 expression in the central amygdala, with slightly lesser expression in the basolateral amygdala. In terms of the PVN, most of the prior IL-6 studies focused on the hypothalamus in general and not on the PVN specifically. Kakizaki and colleagues (1999) reported that LPS administration increased IL-6 expression in the PVN. Vallieres and Rivest (1999) also report that mice that were pretreated with LPS and later treated with IL-6 demonstrated enhanced CRF expression in the PVN. Benrick and colleagues (2009) reported that IL6 KO mice showed diminished expression of CRF in the PVN, and that IL-6Rα was co-expressed with CRF in the PVN. IL-6 in the hypothalamus was found to be increased due to exposure to a variety of stressors including footshocks, hypoxia, chronic unpredictable stress, and restraint (Jankord, et al., 2010; Girotti, et

al., 2013). In a separate study, the forced swimming stressor was found to increase IL-6Rα in the hypothalamus of stressed mice (Aniszewska, et al., 2015).

In support of our findings of the ethanol induced IL-6 expression effects in the PVN and the CEA, a contemporary research group found similar findings with IL-6 mRNA in response to an extended ethanol administration paradigm. While these experiments were in their finishing stages in the Thiele/Lysle lab collaboration, Doremus-Fitzwater and colleagues (2014, 2015) reported in two separate studies that an extended regimen of intraperitoneal or intragastric doses of ethanol will induce enhanced IL-6 mRNA in the amygdala and the PVN. Notably, these results were determined by the use of an alternative technique known as reverse transcription polymerase chain reaction which reports mRNA expression, but does not allow precise spatial resolution to confirm that the expression of IL-6 is most likely due to central amygdala contribution and not due to adjacent regions of the amygdala, such as the basolateral amygdala. There are other notable differences between this group's and our group's findings. For example, intraperitoneal and intragastically applied ethanol represent forced ethanol exposure versus the greater face validity found with the voluntary consumption in our DID paradigm. Furthermore, our DID paradigm would have likely generated lower BECs due to the rodent's propensity to limit consumption when its desired intoxication level is reached. Thus, our results would have been valid at an even lower ethanol exposure than the forced ethanol administration paradigms. Also, our immunochemistry experiments demonstrate the novel finding that IL-6 protein is also increased in response to repeated cycles of binge-like ethanol consumption. Despite technical differences, this contemporary research group's findings support our research. Together, these results indicate that IL-6 signaling in the central amygdala (CEA) and the paraventricular

nucleus of the hypothalamus (PVN) may be involved in the progression or maintenance of binge-like ethanol drinking.

The following study, described in the next chapter, explored the role of IL-6 in binge-like ethanol drinking by antagonizing IL-6R in the CEA or the BLA during the third week of the DID procedure. IL-6R antagonism in the CEA or the BLA was also applied in the third week of the DID procedure with sucrose to establish the extent of IL-6R's role in the consumption of an alternative salient reinforcer.

Figure 2.1 Ethanol, sucrose, and water consumption data. (**A**) Binge-like ethanol consumption (g/kg) during 2-hour access to 20% ethanol on days 1-3 of the DID procedure and during 4-hours of ethanol access on day 4 during the Binge Test. On day 4, mice exposed to 1 or 3 weeks of the DID procedure consumed ~5 g/kg of ethanol over the 4 hour test. This level of consumption is associated with BECs of ~100mg/dl (Lowery-Gionta et al., 2012). (**B**) Fluid consumption (ml/kg) during a separate 1 or 3 week DID procedure with 3% sucrose or water. All data are expressed as mean ± SEM.

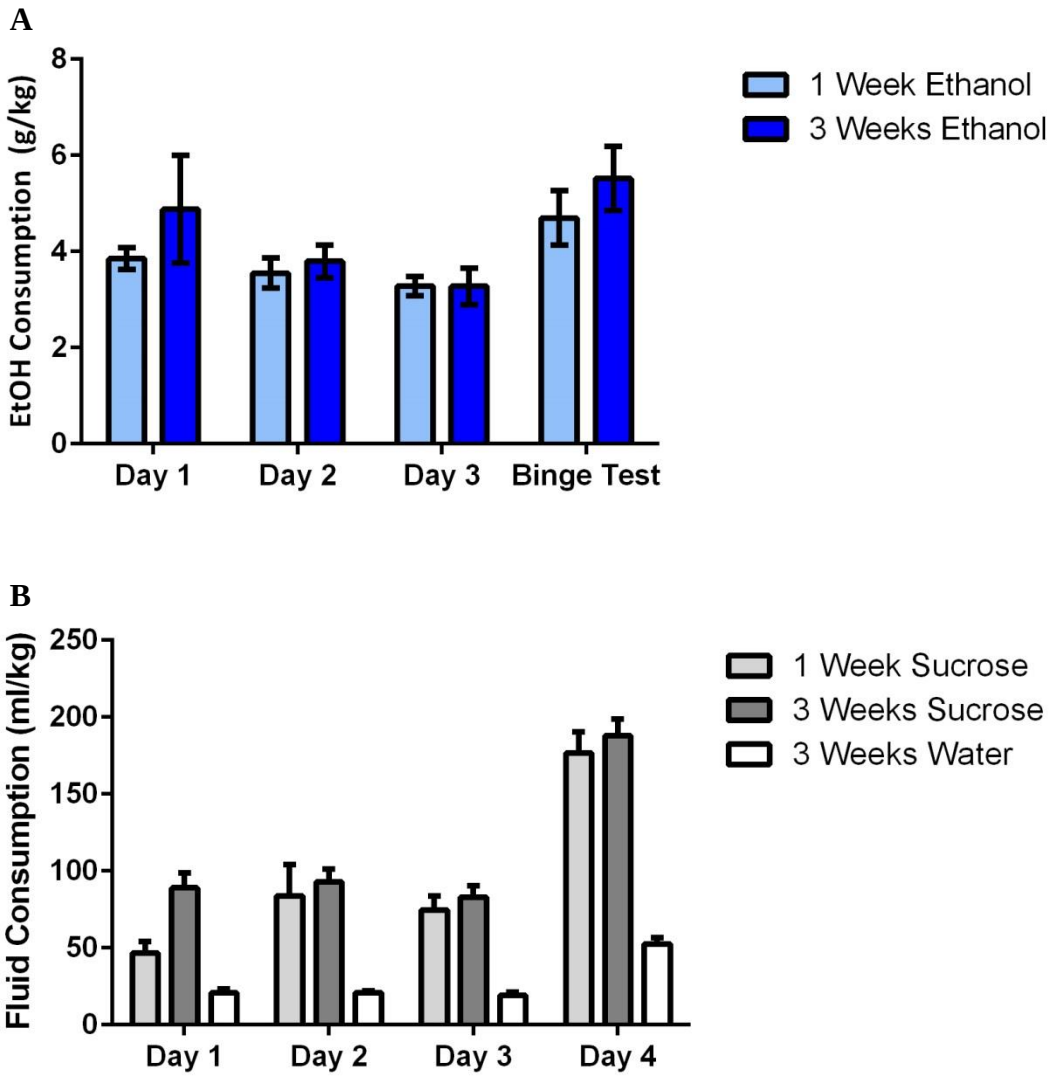
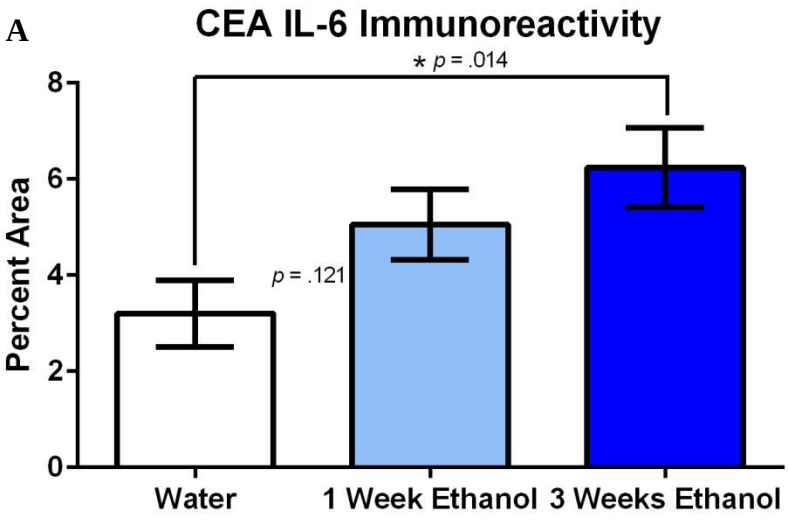
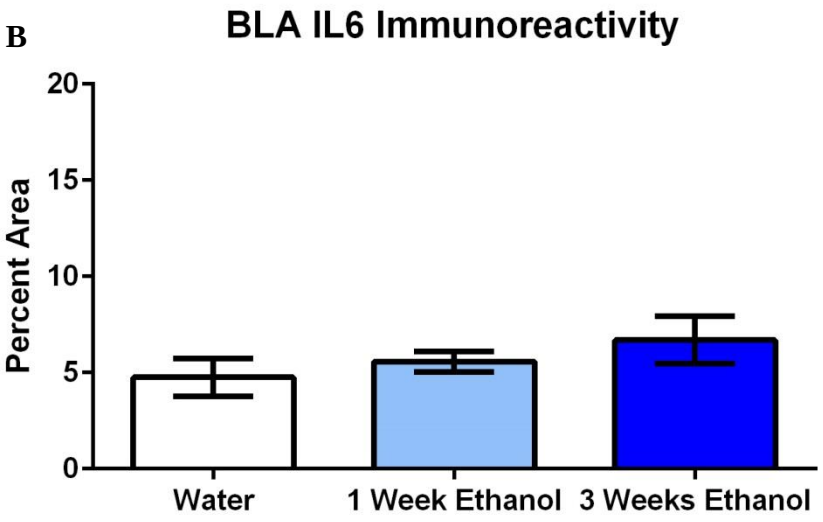


Figure 2.2 Multiple cycles of DID with ethanol are associated with increased IL-6 expression in the central amygdala (CEA). (**A**) Mice exposed to 3 weeks of DID with 20% ethanol showed a significant increase (p=.014) in IL-6 immunoreactivity in the CEA versus water controls. However, there were no significant differences in IL-6 immunoreactivity in the BLA (**B**). (**C**) Mice exposed to 3 weeks of DID with 3% sucrose showed no significant difference in IL-6 immunoreactivity in the CEA versus water controls, suggesting an ethanol specific effect on IL-6 expression at 3 weeks of DID exposure. All data points are expressed as percent area, which is pixels/area in micro meters, and as mean ± SEM.



B


BLA IL6 Immunoreactivity
Percent Area
20
15
10
5
0
Water
1 Week Ethanol
3 Weeks Ethanol

C

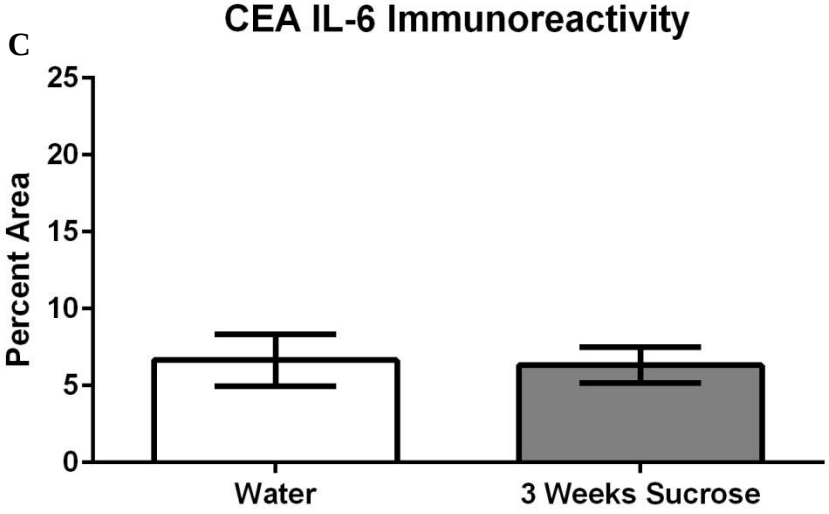
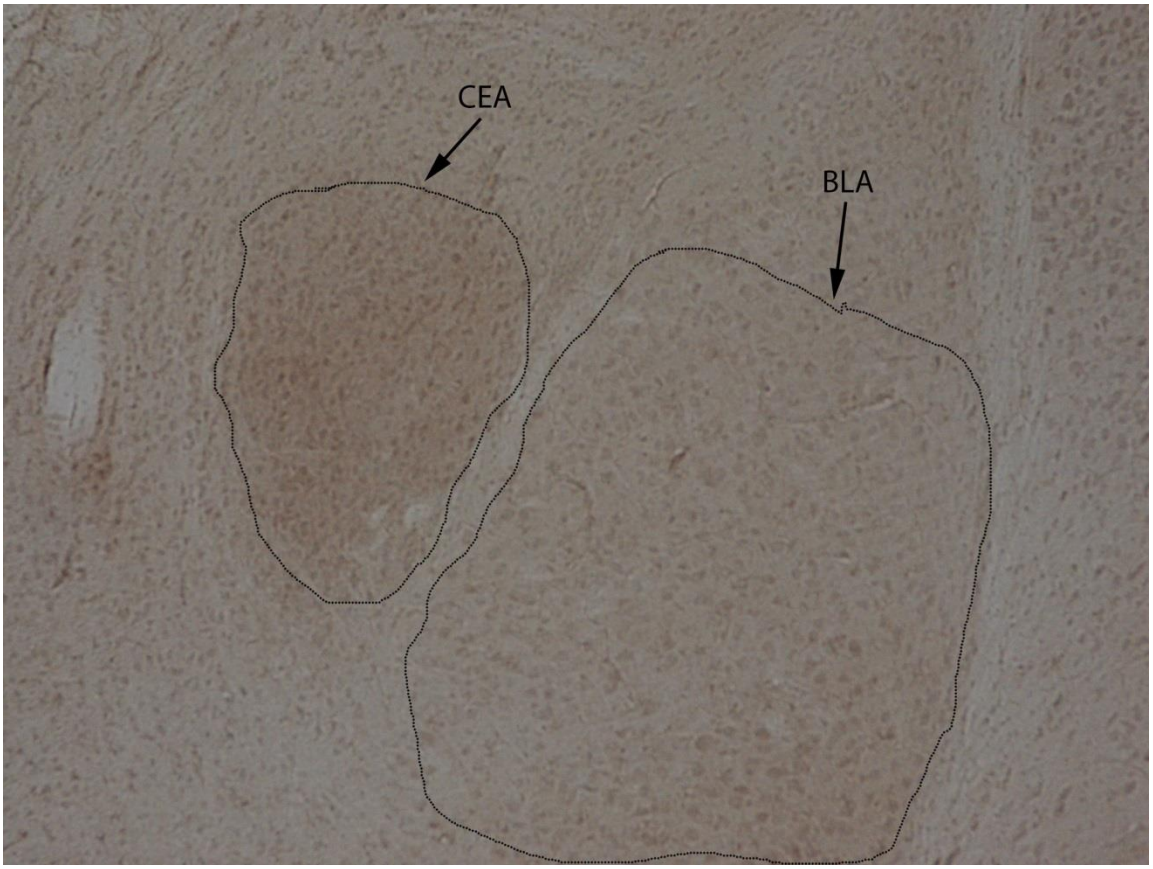

CEA IL-6 Immunoreactivity
Percent Area
25
20
15
10
5
0
Water
3 Weeks Sucrose

Figure 2.3 Representative photographs of IL-6 immunoreactivity in the amygdala. (**A**) Mice exposed to 3 weeks of DID with 20% ethanol show a significant increase in IL-6 immunoreactivity in the central amygdala (CEA) versus water controls (**B**). However, there were no significant differences in IL-6 immunoreactivity in the basolateral amygdala (BLA) (**A**/**B**). Representative photographs of IL-6 immunoreactivity are shown at 100X.

A



B

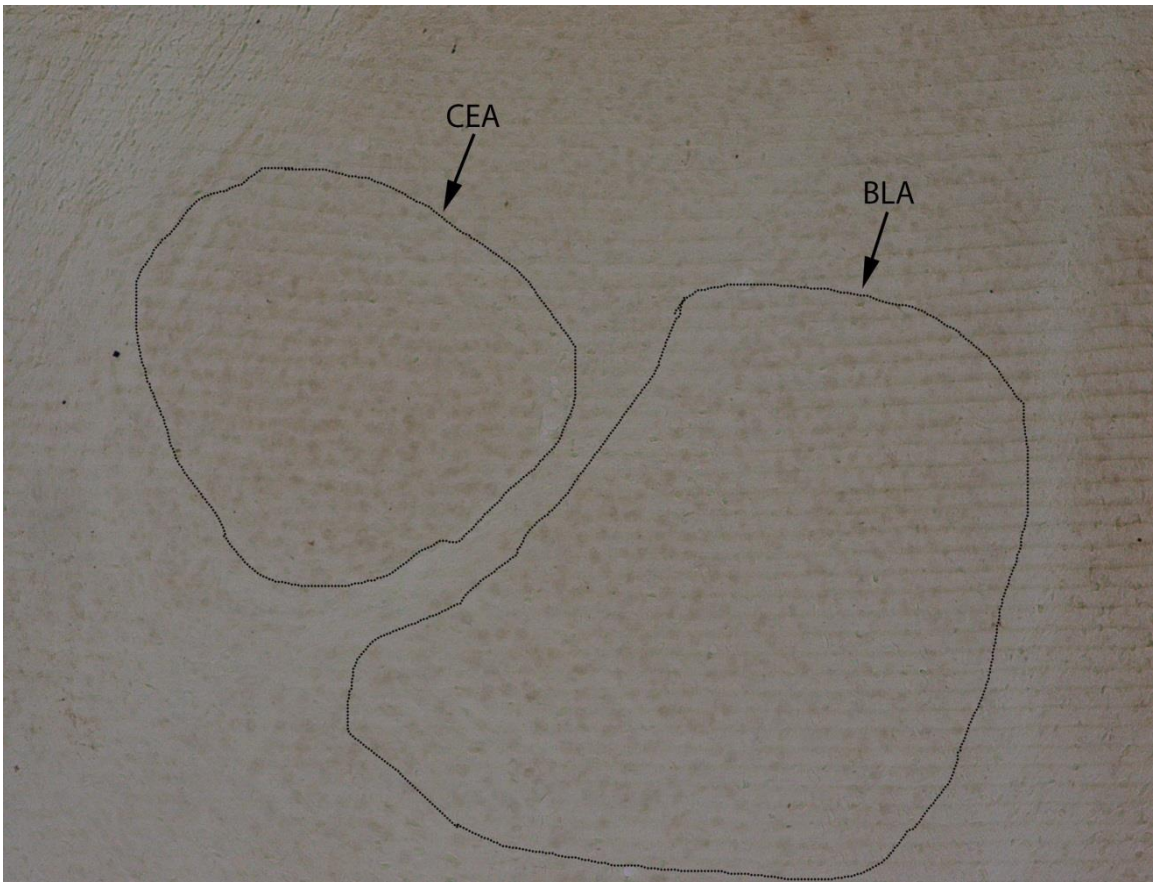
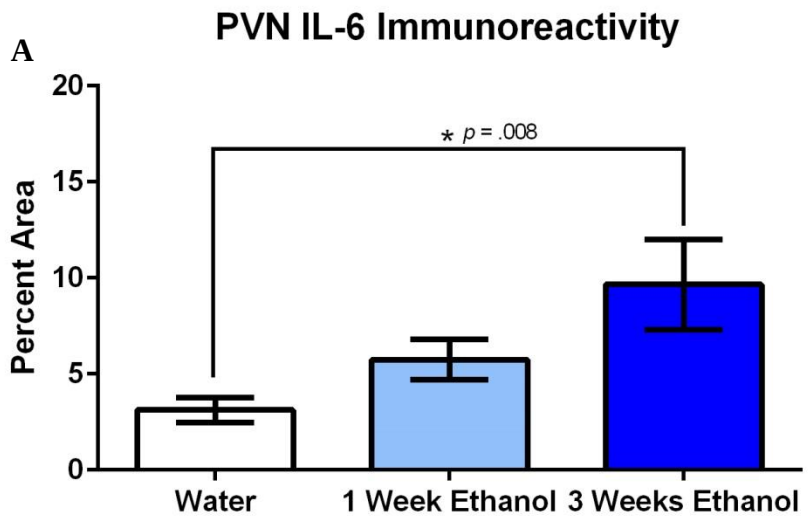


Figure 2.4 Multiple cycles of DID with ethanol are associated with increased IL-6 expression in the paraventricular nucleus of the hypothalamus (PVN). (**A**) Mice exposed to 3 weeks of DID with 20% ethanol showed a significant increase (p=.008) in IL-6 immunoreactivity in the PVN versus water controls. However, there were no significant differences in IL-6 immunoreactivity in adjacent regions of the hypothalamus (data not shown). (**B**) Mice exposed to 1 or 3 weeks of DID with 3% sucrose showed no significant differences in PVN IL-6 immunoreactivity versus water controls, suggesting an ethanol specific effect on IL-6 expression at 3 weeks of DID exposure. All data points are expressed as percent area, which is pixels/area in micro meters, and as mean ± SEM.



B

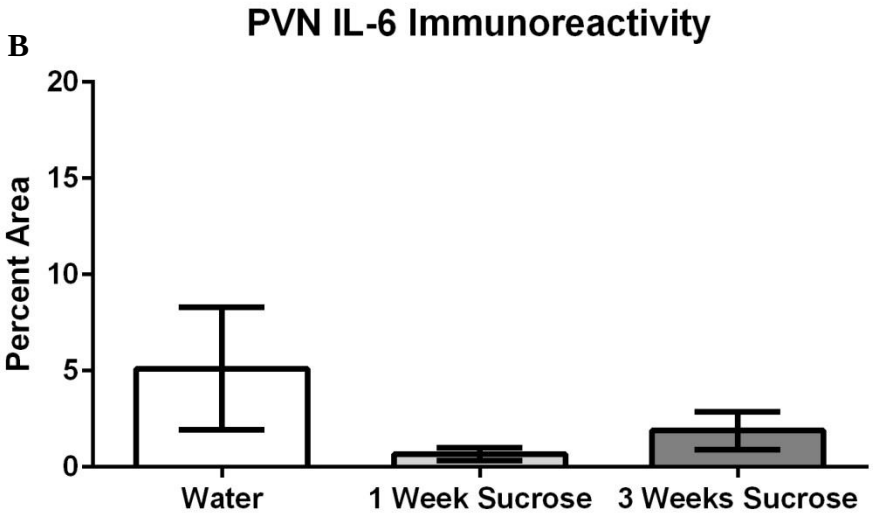
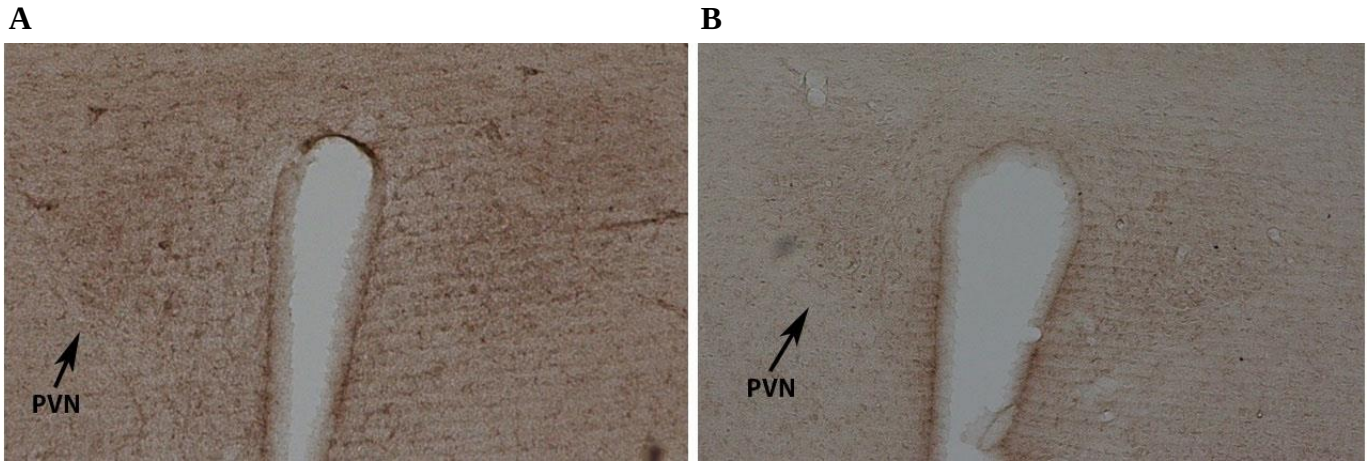

PVN IL-6 Immunoreactivity
Percent Area
20
15
10
5
0
Water
1 Week Sucrose
3 Weeks Sucrose

Figure 2.5 Representative photographs of IL-6 immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN). (**A**) Mice exposed to 3 weeks of DID with 20% ethanol show a significant increase in IL-6 immunoreactivity in the PVN versus water controls (**B**). Representative photographs of IL-6 immunoreactivity are shown at 100X.



Chapter 3

Interleukin-6 receptor antagonism effects on binge-like ethanol consumption

Introduction

Binge ethanol drinking is a risky pattern of drinking that leads to several short and long term consequences, with the most prominent consequence being an increased risk of dependence (Courtney & Polich, 2009). Indeed, there is an accumulation of evidence suggesting that repeated binges alter neurobiological systems to create a stronger motivation to consume ethanol, paralleling the enhanced ethanol consumption and overlapping with the neurobiological changes found in dependence-induced drinking (Sprow, & Thiele, 2012). The neuroimmune system with its associate cytokine actions have been implicated in the neurobiological changes resulting from abusive ethanol consumption. IL-6 is one of the notable cytokines that have been previously implicated in alcohol consumption. For example, genomic microarrays identified IL-6 as a contributor gene to alcohol preference (Mulligan et al., 2006). Deletion of IL-1 and IL-6 genes resulted in reduced ethanol consumption within 2-bottle preference tests (Blednov et al, 2011). In support of ethanol induced IL-6 changes, two studies found ethanol applied to astroglia or microglia in culture caused an increase in IL-6 expression (Boyadjieva, & Sarkar, 2010; Sarc, Wraber, & Lipnik-Stangelj, 2010). Kane and colleagues (2014) report ethanol-induced increases of pro-inflammatory IL-6 mRNA in the cerebellum of C57BL/6J mice in response to oral gavage of 6 g/kg ethanol. Emanuele and colleagues (2005) exposed rats to a chronic ethanol paradigm

which increased TNF-α and IL-6 expression in their hypothalamus. However, these previous studies did not report precisely where the site of action is for IL-6. The sites of action for ethanol induced IL-6 were explored in chapter two of this dissertation, and role of IL-6 in binge-like ethanol drinking is explored further within this chapter (3).

The experiments described within chapter two expand on the role of interleukin-6 by demonstrating that IL-6 immunoreactivity (IR) is up-regulated in the central amygdala and the paraventricular nucleus of the (PVN) hypothalamus of mice with a history of three binge-like ethanol drinking cycles, but not in mice with a comparable history composed of three cycles of sucrose drinking. Notably, this effect was not found in mice that were only exposed to one binge-like ethanol drinking cycle, which suggests IL-6 involvement is more critical after a history of binge-like drinking and could potentially mean that IL-6 might be mediating the neurobiological changes occurring in the overlap between binge-like ethanol drinking and dependence-induced drinking.

Few studies have implicated a role for the PVN of the hypothalamus in binge-like ethanol drinking, but several studies have suggested a role for the central amygdala in binge-like ethanol drinking (Lowery-Gionta et al., 2012; Sparrow et al., 2012). Thus, in pursuit of the more promising target, the present study examined the role of brain IL-6 in excessive binge-like ethanol drinking in C57BL/6J mice, by antagonizing IL-6 receptor actions in key amygdalar brain regions previously implicated in modulating neurobiological responses to ethanol. Mice were exposed to 3 weeks or cycles of the 4 day "drinking in the dark" (DID) binge-like consumption procedure, with 20% ethanol or 3% sucrose in separate experiments.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were approximately 6-8 weeks old and weighed between 20-25g at the beginning of experimental procedures. Mice were individually housed in polypropylene cages with corncob bedding and ad libitum access to standard rodent chow (Purina RMH 3000, Tekland, Madison, WI) and water, except where noted in experimental procedures. The colony rooms were maintained at 22ºC with a reverse 12-hr/12-hr light/dark cycle with lights out at 10 a.m. Mice were run in three cohorts for this study. Cohort 1 consisted of 24 mice used to explore CEA IL-6R antagonism's effects on binge-like ethanol consumption. Cohort 2 consisted of 24 mice used to explore CEA IL-6R antagonism's effects on sucrose consumption. Cohort 3 consisted of 24 mice used to explore BLA IL-6R antagonism's effects on ethanol, as well as sucrose consumption. All experimental procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC) and complied with the NIH Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

Binge-Like Drinking Procedures

The "drinking in the dark" (DID) protocol involves a 4-day procedure and is an animal model of binge ethanol consumption (Rhodes et al., 2005). Throughout the present experiments, mice remain in their home-cages in the vivarium. Beginning 3 hours into the dark cycle, water bottles were removed from the home-cage and replaced with a bottle containing a solution of 20% (v/v) ethanol. On days 1-3, ethanol bottles remained on the cages for 2 hours before removal and replacement with water bottles. On day 4 (the test day), procedures were the same

as on days 1-3 except mice had access to ethanol for 4 hours. Mice were exposed to 3 cycles (weeks) of the DID protocol with ethanol, or 3% sucrose in a separate experiment.

Surgical Procedures:

For cannulae placement surgeries, animals were anesthetized using a cocktail of xylazine (10 mg/kg) and ketamine (100 mg/kg) delivered intraperitoneally (1.5 mL/kg). Bilateral 26 gauge guide cannulae (Plastics One; Roanoke, VA) were aimed at the CEA (AP: -1.06, ML: ±2.50, DV: -4.64) or BLA (AP: -1.22, ML: ±2.9, DV: -4.75) using an Angle II Stereotax (Leica Instruments, Houston, TX) (Paxinos and Franklin, 2001). The measured distance between bregma and lambda were divided by 4.21mm, which is the average distance between these structures. This value was multiplied by the aforementioned coordinates to allow adjusted coordinates for mouse brain size. Coordinate adjustments were similar to previous studies (Moore & Boehm, 2009). After a week of recovery, animals were subjected to the DID procedure for three weeks or cycles. On the test day of week 3, 1-2 hours prior to alcohol (20%) or sucrose (3%) access, 1 ug of IL-6 receptor antagonist (cMR16-1, monoclonal IgG1 neutralizing antibody, Genentech; San Francisco, CA) was dissolved in 0.3ul saline or control infusions of saline alone (0.3 ul) were bilaterally infused at a rate of 0.3 uL/min for one minute using a 1.0 ul Hamilton syringe (Reno, NV) similar to previous studies (Lowery-Gionta et al., 2012). This 1ug cMR16-1 dose was chosen based on the piloting of a few effective doses, and based upon doses used in similar studies (Lowery-Gionta et al., 2012; Marshall et al., 2015). Injectors were left in the guide cannulae for an additional minute to allow proper diffusion away from the injector. Mice were handled and habituated to the infusion procedures at least 2 times prior to test day. Immediately after the 4 hour binge test, tail blood samples (5 µl) were collected to assess BECs with an alcohol analyzer (Analox Instruments, Lunenburg, MA). At the

conclusion of all behavioral analyses injection placements were histologically verified using an identical volume of Alcian blue dye (0.3 uL/injection site) as the IL-6 receptor antagonist experiments. Any animals with unilateral or bilateral misses were excluded from data analyses.

Data Analysis

Two-way analyses of variance (ANOVAs) were used to assess between-group and within-group differences in binge-like ethanol consumption, or binge-like sucrose consumption. Independent samples t-tests were used to determine specific differences in hourly consumption between experimental and control groups. Significance was accepted at $p<0.05$; all data is presented as mean ± SEM. For antagonism data, some animals were excluded from analysis (due to mouse health or unilateral/bilateral cannula misses), which accounts for differences in degrees of freedom between similar analyses.

Results

IL-6R antagonism in the central amygdala (CEA) reduces binge-like ethanol consumption, but does not alter sucrose consumption

Mice in Cohort 1 were exposed to three cycles of the DID procedure with 20% ethanol. There was a significant interaction between hours of consumption and treatment ($F(3,39)=4.090$, $p=.013$), an effect of hours of consumption($F=3,39=5.432$, $p=.003$), and an effect of treatment ($F(1,13)=5.715$, $p=.033$. Essentially, mice who received IL-6R antagonist in the CEA demonstrated significantly reduced ethanol consumption during the first hour ($p=.039$), the third hour ($p=.022$), and in total consumption ($p=.033$) as expressed in grams per kilograms compared to saline treated controls (**Figure 3.1A**). Mice in Cohort 2 were exposed to three cycles of the DID procedure with 3% Sucrose. Mice who received IL-6R antagonist in the CEA demonstrated a non-significant interaction of hours of consumption by treatment ($F(3,60)=1.067$, $p=.370$), and

a non-significant effect of hours of consumption (F(3,60)=2.615, *p*=.059). Most importantly, mice who received IL-6R antagonist in the CEA demonstrated no significant differences in 3% sucrose consumption as expressed in milliliters per kilograms compared to saline treated controls (F(1,20)=.465, *p*=.465), suggesting that IL-6R antagonism specifically affects ethanol consumption and not other salient reinforcers with calories **(Figure 3.1B**).

IL-6R antagonism in the basolateral amygdala (BLA) does not alter binge-like ethanol consumption, or sucrose consumption

Mice in Cohort 3 were exposed to three cycles of the DID procedure with either 20% ethanol, or 3% sucrose. Mice who received IL-6R antagonist in the BLA demonstrated a non-significant interaction of hours of ethanol consumption by treatment group (F(3,30)=.813, *p*=.497), and a non-significant effect of hours of consumption (F(3,30)=1.312, *p*=.289). Most importantly, mice who received IL-6R antagonist in the BLA demonstrated no significant differences in ethanol consumption (F(1,10)=.004, *p*=.954), compared to saline treated controls (**Figure 3.2A**). Mice who received IL-6R antagonist in the BLA demonstrated a non-significant interaction of hours of sucrose consumption by treatment group (F(3,30)=.4813, *p*=.745), and a significant effect of hours of consumption (F(3,30)=6.474, *p*=.002). Furthermore, mice who received IL-6R antagonist in the BLA demonstrated no significant differences in sucrose consumption (F(1,10)=.274, *p*=.612), compared to saline treated controls (**Figure 3.2B**).

Discussion

Mice with a history of three binge-like ethanol drinking cycles that received IL-6R antagonist in the central amygdala demonstrated significantly reduced ethanol consumption during the first hour, the third hour, and in total consumption compared to saline treated controls. Mice that received IL-6R antagonism in the basolateral amygdala demonstrated no significant

differences in ethanol consumption as compared to saline treated controls, which suggests that IL-6 actions in the central amygdala but not the basolateral amygdala modulate ethanol consumption. This finding is especially significant because it suggests that possible drug diffusions from infusion site into adjacent regions are not causing this reduction in ethanol consumption. Also, since the basolateral amygdala has been previously found to be a critical region for IL-1R signaling (Marshall et al., 2016) this finding suggests that the basolateral amygdala is not a critical region for IL-6R signaling in the modulation of binge-like ethanol drinking.

Follow up tests were run with sucrose to determine if the role of IL-6R signaling is specific to the modulation of ethanol consumption. Mice that experienced three binge-like sucrose drinking cycles that received IL6R antagonist in the central amygdala or the basolateral did not demonstrate differences in consumption versus saline treated controls, which suggests that IL-6 actions affect ethanol without impacting the rewarding sucrose consumption. This finding is also important for other reasons as well. Since the IL-6R antagonist only affected ethanol drinking when infused in the central amygdala and had no impact on sucrose consumption when infused in either amygdalar region, this effect acts as tool validation for the IL-6R antagonist (neutralizing antibody). If this effect were found with consumption of both fluids or in both regions that were tested, then there might be a possibility that a neutralizing antibody works by non-specifically altering all consumption wherever it is infused. However, the specificity of these IL-6R effects are in line with the known IL-6 actions and would not suggest the neutralizing antibodies are unsophisticated tools for altering IL-6 signaling. Future studies might use control antibodies for infusions as opposed to the (vehicle) saline infusions used within these novel studies, but given the specificity of these effects, alternative controls are not

expected to alter these particular findings. Indeed, both types of controls have been used previously in the literature (Xin et al., 2014; Narkbunnam et al., 2013), and a thorough search of the Pubmed article database revealed no demonstrated effects with control antibody infusions.

MR16-1 neutralizing antibody has been used in a variety of applications since 1993. Tamura and colleagues (1993) reported that MR16-1 was used to demonstrate that IL-6 might play a role with osteoclast formation, which is part of a bone-maintenance process. Yoshida, Hashizume, and Mihara (2011) demonstrated that MR16-1 suppressed the onset of a laboratory induced model of arthritis. Narkbunnam and colleagues (2013) used MR16-1 and found that this drug is effective as an adjunctive therapy in reducing swelling and pathology associated with hemophilia. Arima and colleagues (2014) discovered that MR16-1 is effective for reducing inflammation in a mouse model of spinal cord injury. Also, Fujita and colleagues (2014) used MR16-1 to promote muscle regeneration in mice. A fuller characterization of MR16-1's properties might be found in a paper by Okazaki and colleagues (2002). Indeed, this dissertation study and its associate experiments with IL-6R antagonist represent the first studies to use MR16-1 in the brains of living mice, and the first to explore IL-6R antagonism as a treatment for reducing ethanol consumption.

Within these IL-6R antagonism experiments reported in this dissertation study, there were no observed immune consequences resulting from the cannulation procedure. Specifically, there were no behavioral abnormalities or inflammation attributable to cannulation to report. Additionally, the placement checks revealed no neurobiological anomalies surrounding the cannula tracts. In the basolateral IL-6R antagonism experiments, the mice were alive for approximately two months with no behavioral abnormalities to report from the cannulation or the IL-6R antagonism. One of the hallmark features of sickness is anhedonia, which is the loss of

enjoyment from pleasurable activities (Dantzer et al., 2008). Anhedonia is typically viewed by the reduced pursuit of pleasurable activities. These mice that received IL-6R antagonism in the basolateral or central amygdala demonstrated no reduction in consumption of the very rewarding sucrose solutions found within these experiments, as compared to saline-infused controls. Notably, these mice that received IL-6R antagonism in either amygdalar regions drank at similar levels to the unmanipulated mice drinking sucrose within the immunohistochemistry experiments reported in chapter two.

The fact that mice that received IL-6R antagonism in the basolateral or central amygdala demonstrated no reduction in sucrose consumption within these experiments, as compared to saline-infused controls is a critical finding to note for other reasons as well. IL-6 has been previously investigated in past research for its potential roles in stress processes, neuroendocrine processes, fever and sickness, and immunometabolism. Schobitz and colleagues (1995) administered exogenously generated IL-6 ICV to rodents and discovered that this increase in IL-6 will raise body temperature, reduce locomotor activity, reduce food intake, but not alter water consumption. Lenczowski and colleagues (1999) also report that exogenous IL-6 administered ICV generated an HPA response as demonstrated by increased plasma adrenocorticotropic hormone and corticosterone expression. Furthermore, this research group reported that IL-6 also caused an increase in body temperature, but demonstrated no sickness behaviors. "Sickness behaviors", within this context are referring to reduced social investigatory behavior and reduced locomotor activity. In Pal and colleagues (2014) review article, they describe the finding that increased plasma concentration of IL-6 was found in obese patients, and other supportive findings, but they also describe challenges with establishing the role of IL-6 in immunometabolism. Because IL-6 can be expressed as a muscle generated cytokine or myokine

as well as a cytokine, it becomes difficult to determine which variation of this immune messenger is playing a prominent role in immunometabolism. Within this dissertation study, the fact that IL-6R antagonism did not reduce sucrose consumption suggests that the central amygdala is not altering sucrose consumption due to sickness-induced anhedonia or reducing consumption of either ethanol or sucrose due to a global reduction of fluids with calories as might be found if IL-6 was acting through a purely metabolic process.

The findings discovered and reported within this chapter of the dissertation represent the first experiments to identify a critical brain region where IL-6 modulates binge-like ethanol drinking. These findings greatly extend the previous work on ethanol-induced IL-6 expression and provide a technical advantage over previous studies which used IL-6 KO mice. Yet, these findings beg the question, how does IL-6 alter ethanol consumption? What mechanisms are behind this reduce ethanol consumption?

The central amygdala (CEA) is strongly implicated in both anxiety and alcohol use disorders (Gilpin et al., 2015). NPY actions within the CEA have been demonstrated to alter binge-like ethanol consumption (Sparrow et al., 2012), yet no known study has linked IL-6 to NPY mediated processes. CRF actions within the CEA have also been demonstrated to alter binge-like ethanol consumption (Lowery-Gionta et al., 2012), and CRF actions within the CEA have been implicated in mediating cytokine actions in other studies (Sternberg, 2006). Huang and colleagues (2010) reported that CRF released by stress acts on CRF-1 receptors within the CEA that sensitize anxiety-like behavior during withdrawal from chronic ethanol exposure. A review article by Breese and Knapp (2016) further describes the interactions between stress, anxiety, CRF, and neuroimmune signaling.

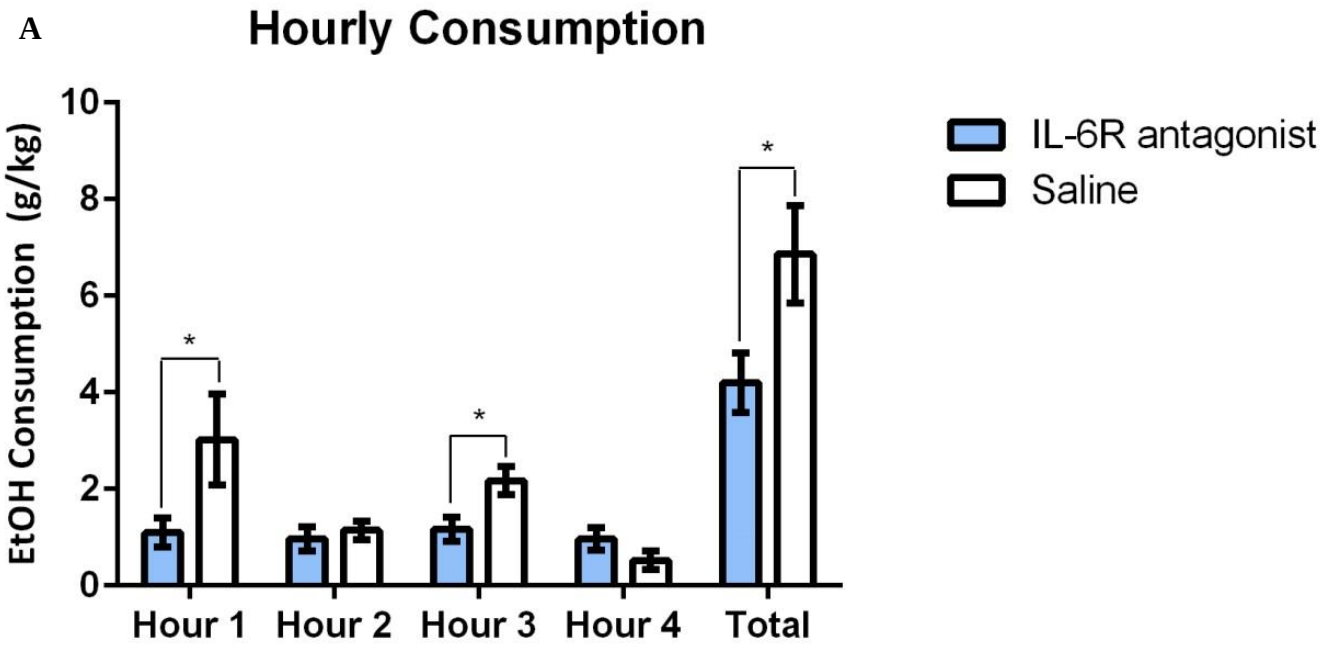
Following several binge exposures to alcohol, the abstinence from alcohol drinking causes an aversive withdrawal state. In this period of negative reinforcement, alcohol is then taken to reduce the aversive effects of the withdrawal experience (i.e., relapse). Cytokines have been implicated in the ethanol withdrawal experience, and IL-6 has been shown to play a role in emotionality, with IL-6 KO mice being more emotionally reactive to stimuli (Armario et al., 1998; Butterweck et al., 2003). However, Heilig and colleagues (2010) note that this emotional component or change in affective processing is longer lasting and potentially more subtle than the anxiety, depression, and negative affect found with acute withdrawal. The assessments within the dissertation experiments described in chapters 2 and 3 occurred immediately after voluntary ethanol consumption. Thus, it is more likely that a potential IL-6 role in an anxiety/stress modulation of ethanol consumption would be due to a change in affective processing and not an acute withdrawal effect.

Alternatively, IL-6 could be interacting with GABA neurotransmission and affecting the rewarding or aversive properties of binge-like ethanol consumption. The central amygdala is primarily GABAergic, and is strongly implicated in both anxiety and alcohol use disorders (Gilpin et al., 2015). In a series of studies using IL-1 KO mice, IL-1 receptor antagonist, and recombinant IL-1ß, Bajo and colleagues (2014, 2015) established a role for IL-1 in the modulation of ethanol-induced GABAergic neurotransmission in the central amygdala. Interleukin-6 has been implicated to interact with GABA, but studies establishing the ethanol contribution to this interaction have not yet been reported (Garcia-Oscos et al., 2012; Gruol, 2015; Hernandez et al., 2016).

Finally, reduced sensitivity to ethanol (as demonstrated by increased sedative behaviors) is often associated with increased ethanol consumption in rodent models (Thiele et al., 2000).

The findings (reported in chapters 2 and 3) that IL-6 is upregulated within the central amygdala in response to three binge-like ethanol cycles, and that antagonism of the IL-6R reduces binge-like ethanol consumption might suggest that cytokines play a role in ethanol sensitivity. The following study, described in the next chapter, explored the role of brain IL-6 in ethanol-induced moderate sedation/ataxia in C57BL/6J mice, by antagonizing IL-6 receptor actions in the central amygdala brain region previously implicated in modulating neurobiological responses to ethanol. Mice were infused with saline or IL-6R antagonist and later exposed to an intraperitoneal 2 g/kg dose of 20% ethanol and tested on rotarod latency. This study was run with the objective to determine whether or not IL-6R antagonism reduces consumption through an alteration of ethanol sedation or sensitivity processes. This investigation into the role of IL-6 in ethanol sensitivity represents one of several possible mechanisms to explain the novel finding that IL-6 can alter binge-like ethanol consumption.

Figure 3.1 Mice received central amygdala (CEA) cannulation and were exposed to three cycles of the DID procedure with either 20% ethanol, or 3% sucrose. (**A**) Mice who received IL-6R antagonist in the CEA demonstrated significantly reduced ethanol consumption during the first hour (p=.039), the third hour (p=.022), and in total consumption (F(1,13)=5.715, p=.033) as expressed in grams per kilograms compared to saline treated controls. (**B**) However, Mice who received IL-6R antagonist in the CEA demonstrated no significant differences in sucrose consumption as expressed in milliliters per kilograms compared to saline treated controls.



B

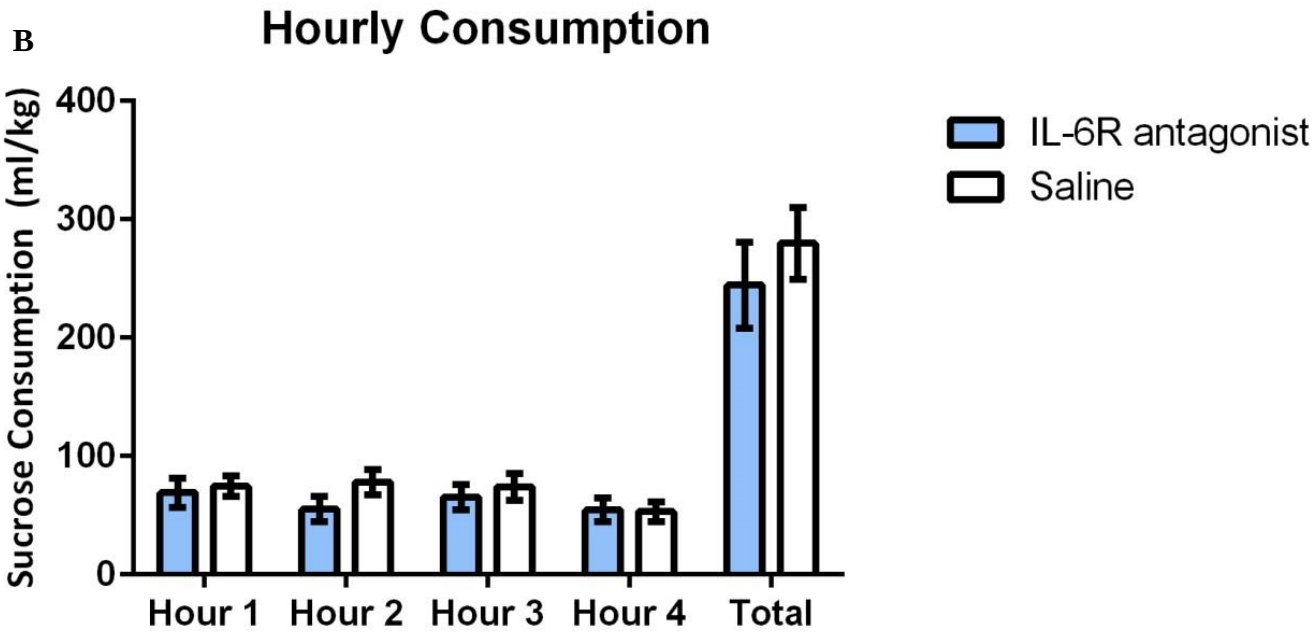
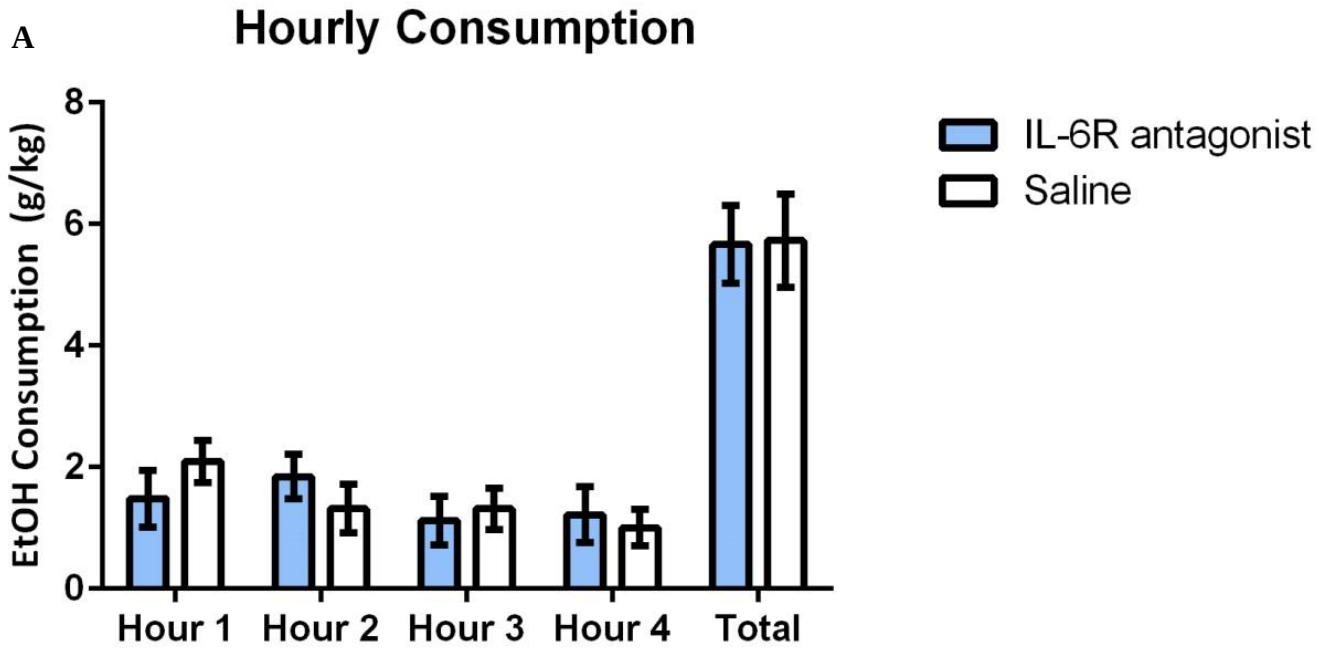

Hourly Consumption
Sucrose Consumption (ml/kg)
400
300
200
100
0
Hour 1
Hour 2
Hour 3
Hour 4
Total
IL-6R antagonist
Saline

Figure 3.2 Mice received basolateral amygdala (BLA) cannulation and were exposed to three cycles of the DID procedure with either 20% ethanol or 3% sucrose. (**A**) Mice who received IL-6R antagonist in the BLA demonstrated no significant differences in ethanol consumption as expressed in grams per kilograms compared to saline treated controls. (**B**) Furthermore, Mice who received IL-6R antagonist in the BLA demonstrated no significant differences in sucrose consumption as expressed in milliliters per kilograms compared to saline treated controls.



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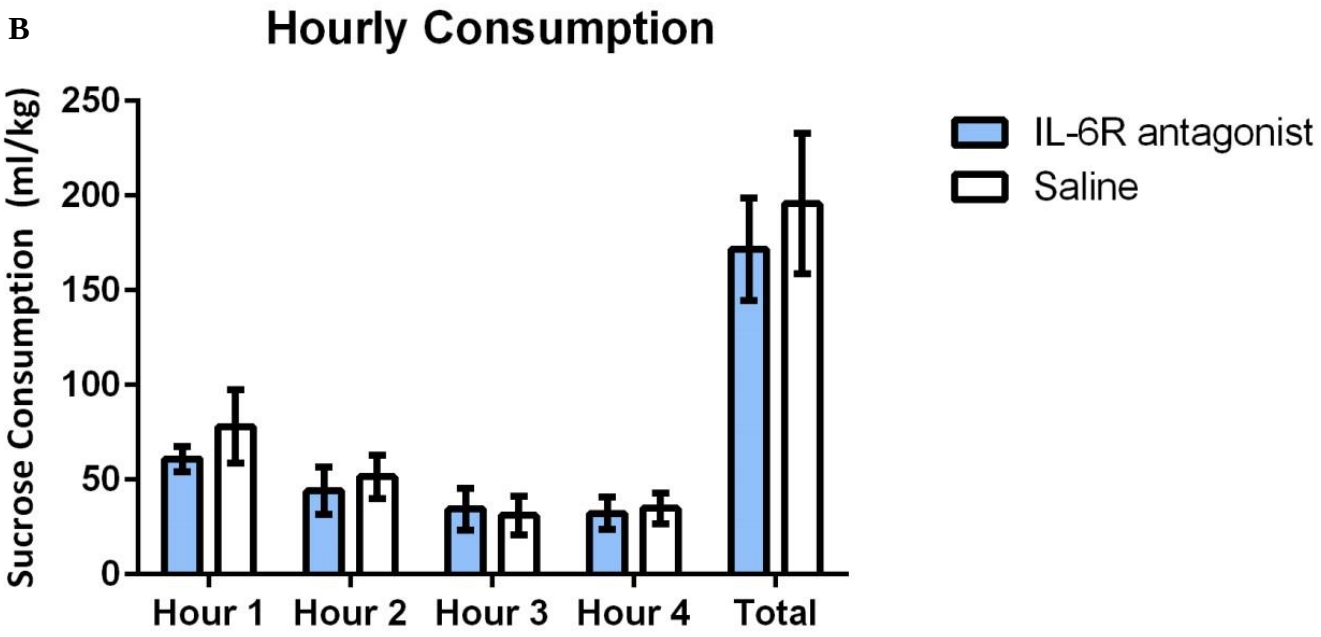

Hourly Consumption
Sucrose Consumption (ml/kg)
250
200
150
100
50
0
Hour 1
Hour 2
Hour 3
Hour 4
Total
IL-6R antagonist
Saline

Chapter 4

Interleukin-6 receptor antagonism effects on moderate ethanol-induced sedation/ataxia

Low level of response or sensitivity to ethanol, as evident by delayed intoxication or sedation, has been associated with high risk for later alcoholism (Shuckit, 1994). Shuckit and colleagues (2011) suggest that the low sensitivity to ethanol may be seen as both a genetic and environmental contributor to alcoholism. The genetic component lies in the fact that approximately 50% of low alcohol response is genetically mediated. The environmental component is due to the higher number of drinks needed to achieve the desired ethanol effects. Paralleling these facts, cytokines have been demonstrated to produce an environmental factor in ethanol drinking, (since ethanol drinking induces cytokines, see Chapter 2), and cytokine gene polymorphisms have been implicated with higher incidences of alcohol dependence (Liu et al., 2009; Gonzalez et al., 2008). Furthermore, reduced sensitivity to ethanol is often associated with increased ethanol consumption in rodent models (Thiele et al., 2000). Thus, this converging evidence suggests that pro-inflammatory cytokines may play a role in ethanol sensitivity.

Cytokines may generate a high sensitivity/sedation due to a potential over-response of cytokines which would then usher in sickness or sedation to cope with the cytokine response. In this scenario, cytokine antagonists would work to normalize the cytokine over-reaction. Wu and colleagues (2011) report that the *high peripheral dose* of 100 mg/kg/i.p. IL-1RA reduced ethanol-induced sedation (sleep time) and motor impairment. Additionally, this research team (2011) also reports that TLR4 KOs and Myd88 KOs (immune gene knock-out mice) show

reduced sedation and motor impairment than matched controls. Corrigan and colleagues (2014) also report that TLR2 KOs show minimal sedation behaviors in comparison to wild-type controls. Yet, high doses of antagonist or gene deletion may not present that best tools for assessing IL-1's actions. Gene knockdown methods are preferable tools to avoid developmental or immune system defects, or compensations from never possessing the gene (KO mice). Also, gene knockdown methods allow temporal and spatial characterization or manipulation of these genes that is not easily achieved with KO mice or peripheral injections.

In contrast to the previously mentioned studies, Vicente-Rodriguez and colleagues (2014) report that mutant mice that overexpress the cytokine pleiotrophin show enhanced ethanol preference and reduced sedation/ataxia in response to ethanol administration. As evident by these aforementioned studies, prior research has provided mixed results concerning the role of cytokines in ethanol sensitivity and sedation. Despite the mixed results regarding cytokines' role in ethanol sensitivity and consumption, based upon previous studies (Shuckit, 1994; Shuckit et al., 2011; Thiele et al., 2000), and the fact that IL-6R antagonist reduced ethanol consumption (chapter 3), it is predicted that IL-6R antagonist will increase ethanol sensitivity.

The present study examined the role of brain IL-6 in ethanol-induced moderate sedation/ataxia in C57BL/6J mice, by antagonizing IL-6 receptor actions in the central amygdala brain region previously implicated in modulating neurobiological responses to ethanol. Mice were infused with saline or IL-6R antagonist and later exposed to an intraperitoneal 2 g/kg dose of 20% ethanol and tested on rotarod latency.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were approximately 6-8 weeks old and weighed between 20-25g at the beginning of experimental procedures. Mice were individually housed in polypropylene cages with corncob bedding and ad libitum access to standard rodent chow (Purina RMH 3000, Tekland, Madison, WI) and water, except where noted in experimental procedures. The colony rooms were maintained at 22ºC with a reverse 12-hr/12-hr light/dark cycle with lights out at 10 a.m. One cohort was used for this study, which consisted of 12 mice that were previously used to explore CEA IL-6R antagonism's effects on sucrose consumption. These 12 mice experienced only control saline infusions during this prior sucrose study. All experimental procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC) and complied with the NIH Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

Surgical Procedures:

Mice were implanted with cannulae and experienced sucrose drinking and infusion procedures as described in chapter 3. At the conclusion of all behavioral analyses (described below) injection placements were histologically verified using an identical volume of Alcian blue dye (0.3 uL/injection site) as the IL-6 receptor antagonist experiments. Any animals with unilateral or bilateral misses were excluded from data analyses.

Sedation/Ataxia Procedures

Accelerating rotarod test

The rotarod apparatus (Ugo Basile Biological Research, Varese, Italy) consisted of a 3 cm diameter horizontal rotating rod divided into five 6 cm sections by tan acrylic disks. The rod was rotated by a motor that accelerated from 0 to 40 rpm over the course of 5 min. For each trial, the mouse was placed on the stationary rod, which was then rotated until the mouse fell. Mice experienced one pre-test day consisting of 5 trials to establish an average baseline performance level for latency to fall from the rotarod. On test day, one hour and 5 minutes prior to the test, mice received either saline (0.3ul) or IL-6R antagonist, cMR16-1 (1ug/0.3ul), infused bilaterally into the central amygdala. Five minutes prior to rotarod testing, all mice received a 2.0 g/kg i.p. injection of 20% ethanol (w/v) mixed in isotonic saline. This 2.0 g/kg i.p. ethanol dose is based upon Rustay and colleagues' (2003) study. Mice experienced 4 trials on the test day. This study was repeated the following week with flipped groups in a Latin Square design. The rotarod test was used to assess sensitivity to alcohol-induced sedation and motor incoordination (ataxia).

Data Analysis

Two-way analyses of variance (ANOVAs) were used to assess between-group and within-group differences in rotarod latency to fall (averaged over trials) over the course of the two days of rotarod testing. Independent samples t-tests were used to determine specific differences in rotarod latency (averaged over trials) between experimental and control groups on test day. Significance was accepted at $p<0.05$; all data is presented as mean ± SEM. Some animals were excluded from analysis (due to mouse health or unilateral/bilateral cannula misses), which accounts for differences in degrees of freedom between similar analyses.

Results

IL-6R antagonism in the central amygdala (CEA) does not alter moderate sedation/ataxia

The groups of mice that experienced saline infusions and the groups that experienced IL-6R antagonist infusions over the two weeks were clustered by treatment and combined for further analysis. There was a non-significant interaction of days of sedation testing by latin order of exposure over the two weeks by treatment group ($F(1,17)=.818$, $p=.378$). There was a non-significant interaction of days of sedation testing by latin order of exposure over the two weeks ($F(1,17)=3.481$, $p=.079$). There was a non-significant interaction of days of sedation testing by treatment group ($F(1,17)=.052$, $p=.822$). On test day, all mice demonstrated reduced average latency to fall compared to the pre-test day due to the ethanol administration ($F(1,17)= 114.307$, $p<.001$). There was a non-significant interaction of treatment groups over the two weeks ($F(1,17)=.258$, $p=.618$). There were no significant effects of latin order over weeks ($F(1,17)=1.845$, $p=.192$), and no significant effects of treatment group ($F(1,17)=.524$, $p=.479$) (**Figure 4.1**). These effects suggest that IL-6R antagonism in the central amygdala does not alter ethanol's moderate sedative/ataxic properties.

Discussion

Mice underwent one pre-test day of rotarod training, and demonstrated no baseline differences between groups in latency to fall from the rotarod apparatus. On test day, mice received either saline or IL-6R antagonist infused into the central amygdala. Subsequently, all mice received a 2.0 g/kg i.p. injection of 20% ethanol. This i.p. injection dose of ethanol was chosen because this dose would generate similar blood ethanol concentrations as binge drinking in the DID paradigm would generate. Compare the 2.0 g/kg i.p. ethanol dose in Doremus-Fitzwater and colleagues' (2014) study and a cycle of DID in Lowery-Gionta and colleagues'

(2012) study for reference. As would be expected from this injection, all mice showed a reduced latency to fall due to the ethanol injection. This suggests that the mice did experience the mild sedative/ataxic effects of a binge-level dose of ethanol. However, there were no test day differences in latency to fall between the mice that received IL-6R antagonist or saline infusions. These effects suggest that IL-6R antagonism in the central amygdala does not alter ethanol's moderate sedative/ataxic properties. Thus, the mechanisms by which IL-6 modulates binge-like ethanol drinking in the CEA do not appear to involve the modulation of ethanol-induced sedation.

Ethanol drinking has been previously shown to increase pro-inflammatory cytokine expression, and cytokine expression increases ethanol drinking. Based upon a review of the literature, it would seem that pro-inflammatory cytokines have an antagonistic relation with ethanol sensitivity/sedation. Thus, pro-inflammatory cytokines would be expected to lower sensitivity to sedation, and would increase ethanol consumption. Indeed, the anticipated effect was for the cytokine antagonist to increase the sedative properties of ethanol. However, the term sedation covers a spectrum of behaviors that range from the moderate sedation of ataxia to the heavy sedation that would cause a subject to fall asleep. Since the drinking in the dark paradigm generates the same blood ethanol concentrations as a 2.0 g/kg i.p. ethanol dose, and this same dose level is commonly used in ataxia studies (Wu et al., 2011; Vicente-Rodriguez et al., 2014), the moderate sedation found with ataxia seemed like the best sedation behavior to assess.

The drinking in the dark (DID) paradigm is a great paradigm for generating binge level drinking, but it was not the chosen first step for assessing moderate sedation for a few reasons. Individual differences do exist between C57BL/6J mice in their total ethanol consumption, but typically the differences are minor. However, if the mice do not consume ethanol at similar

levels, then the interpretation of the IL-6R antagonist's effects would be potentially confounded. Also, some mice will front load their drinking within their first two hours of their binge test day, while others might periodically consume ethanol over their four hour binge test. To administer the IL-6R antagonist without altering ethanol consumption, the antagonist would need to be administered subsequent to the DID procedures. Thus, the challenge would be giving the IL-6 antagonist after all the mice with their respective drinking patterns have drunken to binge levels. Due to the individual differences in total ethanol consumption and the different ethanol consumption patterns, it seemed like the tidiest method would be to use i.p ethanol injections. If differences were found with this method, then a follow-up test would have used the DID procedure followed by the ataxia assay. However, the sedation assessment tests/trials would have needed to be calibrated since alcohol is typically applied intraperitoneally before tests, and not via the DID paradigm.

The rotarod assay is a great method for assessing moderate sedation and ataxia, with a well-established history of effectiveness. (See Rustay et al., 2002 & 2003; Rhodes et al., 2007; Philibin et al., 2008 & 2012; Cox et al., 2013) However, there are other sedations assays available for assessing the spectrum of sedative behaviors at the various other levels of sedation that exist. There are the direct behavioral observations of locomotor activity within an open field apparatus after an approximate 2.0 g/kg i.p. dose of ethanol (Breese et al., 1984). However, such a method can be mired with problems operationally defining variables, with the possibility of more subjective rather than objective scoring of a spectrum of sedative behaviors. Also, there are balance beam tests as well as grid tests which assess sedative behaviors after an approximate 2.0 g/kg i.p. dose of ethanol. The balance beam tests measures footslips on the beam after ethanol administration. However, there are issues with this assay too, such as finding the right

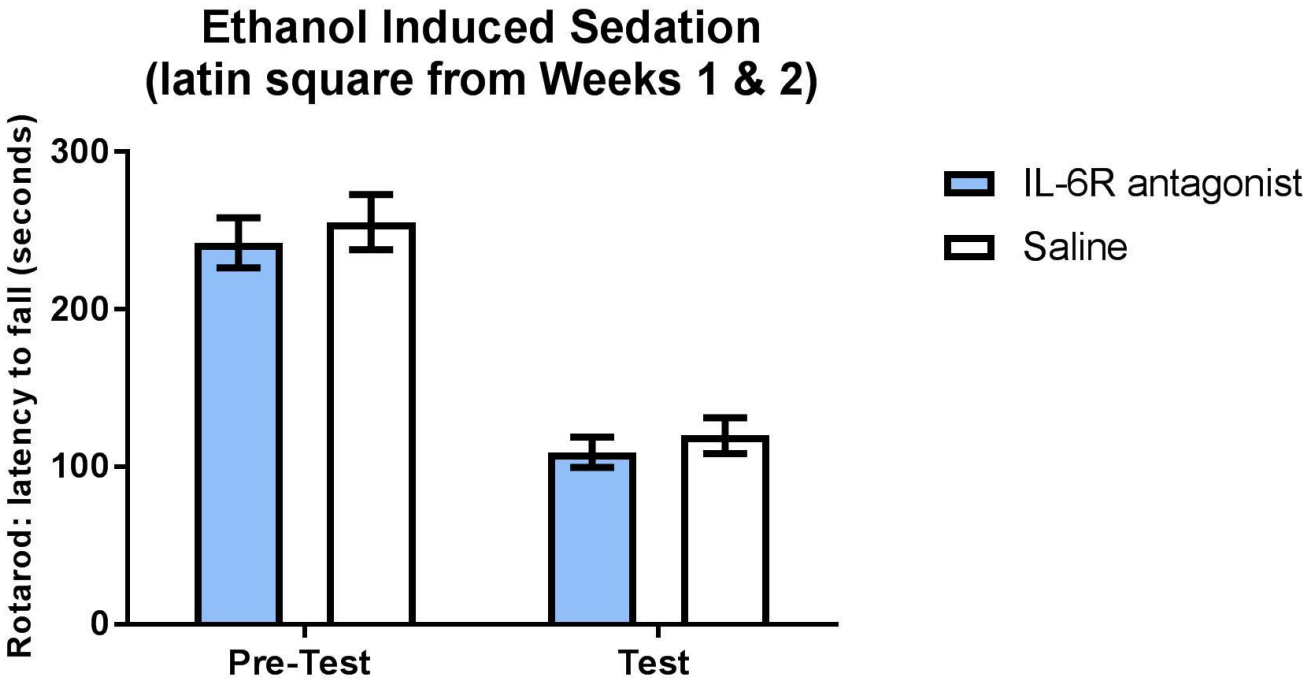
proportions in terms of mouse size in comparison to beam width, defining what constitutes a footslip, and accurately recording the amount of footslips can be challenging for assessments. The grid test is a test in which mice receive ethanol administration and are then placed on a mesh grid, and the mouse is assessed on the amount of time the mouse's foot misses the grid and touches the floor beneath (Crabbe et al., 2003). However, some mice could get their feet caught in the grid, or end up awkwardly positioned due to the unstable grid surface, and end up messing up assessments. These general behavior assessments, balance beam tests, and grid tests are still used, but are typically used as supplemental measures with other sedation assessments. The balance beam test was intended to be used in conjunction with the rotarod tests within this dissertation study, but the negative results found with the rotarod test reduced the need for a supplemental test to further assess moderate sedative behaviors after a binge-level dose of ethanol.

Indeed, the most popular method for studying sedation is through the loss of righting reflex (LORR) task. In this task, mice are typically given a high i.p. dose of ethanol 3.0-4.0 g/kg of 20% ethanol. This dose is well above the consumptions levels in the DID procedure, and will cause the mice to fall asleep. Mice are then placed on their backs, and assessed on the amount of time it takes the mouse to flip over onto its four paws, within a predetermined time period. (See Thiele et al., 2000, 2002, 2003, Fee et al., 2004; Blednov et al., 2005; Wu et al., 2011; Corrigan et al., 2014). This LORR test is an effective test for assessing sedative behaviors, but would appear to be an inappropriate test for determining whether IL-6R modulates binge-like ethanol consumption via the moderate sedation that would be generated by the DID procedure.

In summary, it would appear that IL-6R signaling does not modulate binge-like ethanol consumption via alterations in ethanol sensitivity and its inherent effects with sedation. There are

other modalities that IL-6R signaling could be acting on to impact binge-like ethanol consumption. These modalities will be discussed further within the General Discussion of this dissertation.

Figure 4.1 Mice experienced sedation/ataxia procedures. Mice underwent one pretest day of rotarod training consisting of 5 trials. On test day, one hour and 5 minutes prior to the test, mice received either saline or IL-6R antagonist infused bilaterally into the central amygdala. Five minutes prior to rotarod testing, all mice received a 2.0 g/kg i.p. injection of 20% ethanol. Mice experienced 4 trials on the test day. This study was repeated the following week with flipped groups in a latin square design. All mice demonstrated reduced latency to fall from pre-test day, but demonstrated no significant group differences in test day rotarod performance due to drug treatment.



General Discussion

Interleukin-6 is a pleiotropic cytokine that influences several processes involved with the maintenance and survival of an organism. Within this dissertation, IL-6 has been described as critical cytokine involved with modulating binge-like ethanol consumption. However, IL-6 plays a variety of roles in the body such as regulating inflammation, contributing to neurogenesis, as well as endocrine roles. This cytokine may be produced by neurons, microglia, astrocytes, or endothelial cells. (Erta, Quintana, & Hidalgo, 2012; Hunter, & Jones, 2015). IL-6 is a critical cytokine that controls the transition from innate to adaptive immune processing. However, the roles of IL-6 may be heavily context dependent (Erta, Quintana, & Hidalgo, 2012). IL-6's role may depend on the level of inflammation in the affected regions. IL-6, in concert with sIL-6Rα has been found to modulate the transition between acute and chronic inflammation. IL-6 has been suggested to be protective in low levels, but to be proinflammatory during chronic inflammation. One way that IL-6 has been found to be protective is by inducing production of the anti-inflammatory interleukin-1 receptor antagonist (Gabay, 2006). It would seem by most accounts that IL-6's main goal is to maintain homeostasis in the body.

Interleukin-6 has three signaling pathways that it is known to act on within the body. The least relevant pathway to the topic of neuroimmune signaling is the role that IL-6 plays in the skeletal muscle system. In this system, IL-6 is produced as a myokine which is essentially a cytokine secreted by muscles. In this capacity, IL-6's primarily role appears to be to reduce inflammation and enhance functionality within the muscles, as well as enter the blood stream and

impact the body's metabolism. This, in turn, can have an impact on body mass and is presently investigated in obesity research. In fact, obesity is associated with chronic low-grade inflammation, and so IL-6 might be impacting this chronic inflammation. As a myokine, IL-6 follows a different pathway of signaling and expression than as the cytokine IL-6 (Pal, Febbraio, & Whitham, 2014; Guijarro, Laviano, & Meguid, 2006).

The two most relevant pathways that IL-6 acts on within the neuroimmune system are the classic signaling and the trans-signaling pathways. Classical signaling is when IL-6 binds to IL-6R and gp130. Classical signaling helps maintain homeostasis and can be neuroprotective. In neurons, IL-6 can act as a neuromodulator. IL-6 has been found in the PVN and other regions of the hypothalamus, the hippocampus, and also the cerebellum. (Benrick, et al., 2009; Sallmann, et al., 2000; Aniszewska et al., 2015; Jankord et al., 2010). IL-6 has been detected in both cholinergic and GABAergic neurons. IL-6 is considered a neuropoietic cytokine because it promotes neuronal survival. Additionally, IL-6 has been implicated in assisting long-term potentiation, improving spatial learning, as well as maintaining and modulating neurogenesis (Bowen, et al., 2011; del Rey et al., 2013; Juttler, Tarabin, & Schwaninger, 2002). Depending on the concentrations of IL-6, this cytokine can protect against NMDA excitotoxicity, which is a factor in chronic alcohol drinking. IL-6 may influence neurons directly, but may also act as a messenger between glia and neurons (Juttler, Tarabin, & Schwaninger, 2002). In fact, some preliminary research suggests that IL-6 may act pre-synaptically or post-synaptically to alter neurotransmitter release (Gruol, 2015; Crowley, Cryan, Downer, & O'Leary, 2016).

Trans-signaling is when IL-6 binds with sIL-6R and gp130. Trans-signaling is largely responsible for chronic inflammation (and depression) since the pervasiveness of these binding elements allow IL-6 to affect cells that do not express IL-6R. In fact, IL-6 has also been

demonstrated to be a critical cytokine that modulates sickness behaviors such as reduction of social exploration, loss of body weight, and immobility (Bluthe et al., 2000). Selective antagonists that target IL-6 trans-signaling have been demonstrated to reduce sickness behaviors (Burton et al., 2013), and improve survival rates in mouse sepsis models (Barkhausen et al., 2011). Trans-signaling has been suggested to be involved in gut permeability, which is one of the proposed ways that binge ethanol exposure has been proposed to cause cytokines to circulate and impact the neuroimmune system. Soluble gp130 is one of the endogenous antagonists to this trans-signaling. This antagonist works by competing with the gp130 protein for binding to the IL-6 complex (Maes, Anderson, Kubera, & Berk, 2014; Jostock et al., 2001).

Exploring the therapeutic potential of Interleukin 6 antagonism

Given the potential roles of interleukin 6 (IL-6) in neuroimmune disorders and in binge drinking behaviors, IL-6 antagonism presents a promising method of reducing inflammation and binge-like drinking behaviors. However, potential strategies to target IL-6 signaling should consider whether the protective neurotransmitter-like, homeostatic classical IL-6 system will be impacted or if the pro-inflammatory trans-signaling IL-6 system will be impacted by these interventions. One antagonist that has been prominently featured is tocilizumab (also known as MRA), which is an antibody that targets the IL-6 receptor. This drug is often prescribed as treatment for arthritis and other chronic inflammatory diseases. This drug inhibits both classical and trans-signaling. Another compound that is currently under investigation is the engineered sgp-130fc protein which prevents gp-130 from binding to the IL-6 complex by competitively binding with the IL-6 complex, much like the aforementioned endogenous sgp130 antagonist. In turn, this antagonist only blocks the IL-6 trans-signaling and not the classical IL-6 signaling (Hunter, & Jones, 2015; Jostock et al., 2001). This sgp-130fc compound inhibits only trans-

signaling at low concentrations, but will inhibit classical signaling as well at high concentrations (Garbers et al., 2011). However, at the time of this writing neither compound was available for research purposes.

Finally, the third major compound under investigation is MR16-1 (also known as BP-5875). Okazaki and colleagues (2002) report that MR16-1 interferes with IL-6's binding to the soluble IL-6 receptor by competitively binding to this receptor, which is the principal receptor active during trans-signaling. However, this compound may have the potential to neutralize membrane bound IL-6 receptors as well. MR16-1 has been used previously in studies of arthritis and other chronic inflammation diseases. In fact, MR16-1 has been used to treat mouse models of spinal cord injury. This drug has been shown to reduce inflammation to spare tissues, and decrease the possibility of glial scar formations within this mouse model of spinal cord injury (Arima et al., 2014). Given the accessibility of this compound, and this compound's preferential action on trans-signaling, a variation of this compound was used here for studies exploring the role of IL-6 in alcohol consumption and sedation behaviors. The version of this compound used for these studies was a chimeric MR16-1 (cMR16-1) which was determined to be especially effective in mouse models and will not cross-react with human IL-6R (Fujita et al., 2014).

The challenges of site-specific antagonism

The blood-brain-barrier is an effective barrier for preventing pathogens from entering the brain, but this barrier also presents a significant challenge for drug therapies to enter and affect behavioral disorders. Several approaches to central nervous system drug delivery have been proposed and explored throughout the years, with varying costs and other considerations, as is described by William Banks (2016). While drug delivery still remains a challenge, in the laboratory setting there is one method that has a long, proven history of effectiveness with

laboratory animals, which is the direct infusions of drug into the brain via implanted cannulas. In 1994, Bluthe and colleagues used interleukin-1 receptor antagonist (IL-1RA) administered intracerebroventricularly (ICV) in CD1 mice to reduce the deficits in social interactions caused by prior infusion of TNF-α. Maier and Watkins (1995) also used ICV IL-1RA to block fear conditioning in Sprague-Dawley rats. Goshen and colleagues (2007) used ICV IL-1ß or IL-1RA on mice with a C57 background to demonstrate that a slight increase in IL-1ß will improve contextual memory in a fear conditioning paradigm, but too little or too much IL-1ß will impair contextual memory. Arakawa and colleagues (2009) used IL-1RA to rescue the reduction in social behavior that results from a prior foot-shock stressor. Also in 2009, Moore and Boehm used site-specific infusions of the GABA-B agonist, baclofen, into the anterior ventral tegmental area (VTA) to reduce binge-like ethanol consumption of C57BL/6J mice within the DID paradigm. This group discovered that these infusions will not affect water consumption, nor will the infusions in the adjacent posterior VTA affect binge-like ethanol consumption. Also, this group generated a formula with the assistance of a mouse brain atlas to adjust for the drift in the stereotaxic brain coordinates that may occur due to an individual mouse's unique features, or the drift that may occur due to age-related brain/skull growth.

The Thiele lab has used similar methods in their work exploring CRF actions in binge-like ethanol drinking. This group has found that antagonism of the CRF 1 receptor reduced binge-like ethanol drinking in C57BL/6J mice when this antagonist is infused in the central amygdala but not the basolateral amygdala (Lowery-Gionta, et al., 2012). In fact, recently this group has performed a study exploring the role of IL-1ß in binge-like ethanol drinking. In contrast to the CRF findings, IL-1 receptor antagonism via IL-1RA in the basolateral amygdala, but not the central amygdala reduced binge-like ethanol drinking of C57BL/6J mice within the

DID paradigm. Additionally, IL-1RA did not alter sucrose drinking or open-field activity. Notably, Fluoro-Jade® C labeling indicated that multiple binge-like alcohol cycles did not cause neuronal death suggesting that cytokines actions are not modifying behavior by killing neurons (Marshall, et al, 2015).

All of these previous studies in other neuroimmune labs used cannulation, with no reports of lasting immune consequences of the neurobiological or behavioral nature resulting from the cannulation procedure. Other laboratories that use bilateral cannulations have not reported any lasting immune consequences. For example, Xin and colleagues (2014) used BDNF-neutralizing antibody site-specifically to demonstrate a critical role of BDNF in the basolateral amygdala for conditioned taste aversion. Most importantly, our lab did not observe immune consequences in our prior study which used bilateral cannulas directed at the central or basolateral amygdala. However, while our laboratory and others have not specifically experimentally tested for immune consequences from cannulation, there has been no report of sickness or other noticeable immune actions (site inflammation) resulting from this manipulation. Our study used IL-1RA directed at both of these amygdalar regions to determine that the basolateral amygdala was the critical region for IL-1R mediated ethanol consumption. Also of note, IL-1RA infusion into the basolateral amygdala did not alter sucrose consumption or open-field locomotor activity. These negative results in the open-field assay suggest that IL-1RA is not affecting general locomotor activity or the mouse anxiety this assay is designed to assess (Marshall et al., 2016). Indeed, cannulation may generate a transient immune response, but this would be resolved by allowing mice to have at least a week of recovery prior to further stimulus. Not surprisingly, a week of recovery is a fairly standard practice for labs that perform surgeries.

IL-6 antagonism in the brain is still a fairly new strategy that has not been explored in great detail until this dissertation. Noda and colleagues (2013) used a monoclonal antibody against human IL-6R (known as tocilizumab) in a mouse model which encouraged brain tumor growth in nude mice. This antibody was applied intravenously for twice a week for three weeks, which effectively reduced the tumor size compared to mice exposed to human IgG antibody control infusions. Burton and colleagues (2013) is the only other group known to use central IL-6 antagonism as a strategy for exploring IL-6 actions in the brain. This group used soluble gp130, a known inhibitor of IL-6 trans-signaling, which they infused ICV to reduce LPS induced neuroinflammation and sickness behaviors in aged BALB/c mice.

Summary of Current Findings

Within the immunohistochemistry studies reported here, there were several discoveries on the expression of IL-6 in response to sucrose or ethanol consumption. In fact, relative to water drinking controls, IL-6 immunoreactivity (IR) is up-regulated in the central amygdala and the paraventricular nucleus of the (PVN) hypothalamus of mice with a history of three binge-like ethanol drinking cycles. This effect is not found in mice that were only exposed to one binge-like ethanol drinking cycle. In a separate study comparing sucrose versus water drinking induced IL-6 IR, no significant differences with IL-6 IR were found in the central amygdala or the PVN. This sucrose consumption finding suggests that IL-6 has unique actions in response to ethanol consumption, which are not found with consumption of other salient reinforcers. Additionally, IL-6 IR is unaffected in the (adjacently located) basolateral amygdala of mice with a history of three binge-like ethanol drinking cycles. Notably, IL-6 IR is not increased in the BNST or the nucleus accumbens in response to one or three cycles of binge-like ethanol drinking. This seems to indicate that IL-6, in response to binge-like ethanol drinking, is not altered in these regions of

the reward circuitry indicated in Koob's (2003) allostatic model of alcohol addiction. Also, IL-6 IR is not increased in the lateral septum in response to one or three cycles of binge-like ethanol drinking. The lateral septum is a non-classical reward related region of interest that has been regarded as important for ethanol consumption as described by Ryabinin, Bachtell, and colleagues (2003, 2003, & 2008). Additionally, Breese and colleagues (1984) described the septum as a region that is important for mediating ethanol induced motor impairment or sedative behaviors. However, the significant effects in the central amygdala and the PVN suggest that increased IL-6 expression in the brain is site-specific in response to alcohol consumption. The fact that three cycles or weeks of ethanol exposure, and not one week of ethanol exposure, are needed to produce the enhanced IL-6 expression over the water controls suggests that IL-6 actions are more relevant in subjects with an extended history of binge-like ethanol consumption.

The IL-6R antagonism studies revealed that mice with a history of three binge-like ethanol drinking cycles that received IL-6R antagonist in the central amygdala demonstrated significantly reduced ethanol consumption during the first hour, the third hour, and in total consumption compared to saline treated controls. Mice that received IL-6R antagonism in the basolateral amygdala demonstrated no significant differences in ethanol consumption as compared to saline treated controls, which suggests that IL-6 actions in the central amygdala but not the basolateral amygdala modulate ethanol consumption. This finding is especially significant because it suggests that possible drug diffusions from infusion site into adjacent regions are not a factor. Also, since the basolateral amygdala has been previously found to be a critical region for IL-1R signaling (Marshall et al., 2016) this finding suggests that the basolateral amygdala is not a critical region in IL-6R signaling.

Follow up tests were run with sucrose to determine if IL-6R signaling will only critically modulate ethanol consumption. Mice that experienced three binge-like sucrose drinking cycles that received IL6R antagonist in the central amygdala or the basolateral did not demonstrate differences in consumption versus saline treated controls, which suggests that IL-6 actions affect ethanol without impacting the rewarding sucrose consumption. This finding is also important for other reasons as well. Since the IL-6R antagonist only affected ethanol drinking when infused in the central amygdala and had no impact on sucrose consumption when infused in either amygdalar region, this effect acts as tool validation for the IL-6R antagonist (neutralizing antibody). If this effect were found with consumption of both fluids or in both regions that were tested, then there might be a possibility that a neutralizing antibody works by non-specifically altering all consumption wherever it is infused. However, the specificity of these IL-6R effects are in line with the known IL-6 actions and would not suggest that neutralizing antibodies are unsophisticated tools for altering IL-6 signaling.

The IL-6R antagonism studies also explored the role that IL-6 might play in moderate ethanol-induced sedation. In these studies, mice underwent one pre-test day of rotarod training, and demonstrated no baseline differences between groups in latency to fall from the rotarod apparatus. On test day, mice received either saline or IL-6R antagonist infused into the central amygdala. Subsequently, all mice received a 2.0 g/kg i.p. injection of 20% ethanol. This i.p. injection dose was chosen because this dose would generate similar blood ethanol concentrations as binge drinking in the DID paradigm would generate. As would be expected from this injection, all mice showed a reduced latency to fall due to the ethanol injection. This suggests that the mice did experience the mild sedative/ataxic effects of a binge-level dose of ethanol. However, there were no test day differences in latency to fall between the mice that received IL-

6R antagonist or saline infusions. To confirm this effect, this study was repeated the following week with flipped groups in a Latin Square design. In week two, there were no baseline differences in latency to fall on pre-test day. On test day, all mice demonstrated reduced latency to fall compared to the pre-test day due to the ethanol administration, but demonstrated no significant group differences in test day rotarod performance due to the drug treatment.

The groups that experienced saline infusions and the groups that experienced IL-6R antagonist infusions over the two weeks were then clustered by treatment and combined for further analysis. The combined groups demonstrated no baseline differences on latency to fall on pre-test day. On test day, all mice demonstrated reduced latency to fall compared to the pre-test day due to the ethanol administration, but demonstrated no significant group differences in test day rotarod performance due to drug treatment. Despite the switching of groups, and the second dose of ethanol a week later, there were no group differences in ethanol induced sedation/ataxia. These effects suggest that IL-6R antagonism in the central amygdala does not alter ethanol consumption by altering ethanol's moderate sedative/ataxic properties.

Potential cytokine roles during the escalation of binge alcohol drinking into alcohol dependence

In consideration of the cytokine roles in the brain in response to normal and/or ethanol modulated processes, there is the implication that pro-inflammatory cytokine expression increases binge-like ethanol drinking. One model that has been postulated to account for the actions of the central cytokines within the pathway to dependence or addiction is the Allostasis model developed by Dr. George Koob. Homeostasis is characterized by the bodily processes that work to maintain the functionality and survival of an organism. Allostasis refers to the process where the same adaptive processes that work within homeostasis become dysregulated and these

adaptive processes change to attain stability, yet these changes push the regulatory systems outside the normal set-point into a potentially pathological set-point (Koob, 2003; Koob & Le Moal, 2001). Potentially the impairment in the homeostatic role of IL-6 might be responsible for the inability of the homeostatic mechanisms to attain the original normal set-points.

The descent into alcohol addiction is characterized by experiences of positive and negative reinforcement. At first, alcohol activates the brain reward systems and generates a pleasurable experience. This period of positive reinforcement causes the alcohol user to binge drink alcohol in pursuit of the initial pleasures of alcohol use. Yet, as the body adapts to the continued binge exposures of alcohol, the body does not respond in the same ways to this drug. After several binge exposures to alcohol, cessation of alcohol drinking (abstinence) causes the aversive withdrawal state. In this period of negative reinforcement, alcohol is then taken to reduce the aversive effects of the withdrawal experience (i.e., relapse). Cytokines have been implicated in the ethanol withdrawal experience, and Breese and colleagues (2008) demonstrated that cytokine pre-exposure can sensitize this ethanol-induced anxiety experience. Notably, IL-6 has been shown to play a role in emotionality, with IL-6 KO mice being more emotionally reactive to stimuli (Armario et al., 1998; Butterweck et al., 2003). However, IL-6 expression has also been found to increase in the hippocampus of C57BL/6J mice in response to stressors that are used for depression tests (Chourbaji et al., 2006). Whether or not IL-6 is interacting with emotions or stress responses, independently or together, in reaction to alcohol is yet to be determined.

Conceptually, negative affect or emotional states could drive the descent into alcohol addiction. However, Heilig and colleagues (2010) note that this emotional component or change in affective processing is longer lasting and potentially more subtle than the anxiety, depression,

and negative affect found with acute withdrawal. Frequent binges followed by their withdrawals have been found to exacerbate the acute withdrawal effects which have been demonstrated to cause the alcohol abuser to increase voluntary ethanol consumption as well as become more reactive to stress. Yet, measuring the anxiety that is correlated with these phenomena is complicated and will likely need multiple assays to provide concrete answers on the anxiety roles. The subtle neuroadaptations underlying this process have been suggested to be related to HPA activation or CRF activity. Notably, stress has been found to be an adequate substitute for multiple binge cycles (Breese et al., 2005). These findings suggest that anxiety and stress might both by acting on binge alcohol drinking behaviors.

In support of these stress roles in ethanol consumption, Knapp and colleagues (2011) describe a series of experiments exploring the cytokine interactions with stress and alcohol consumption. Restraint stress elevated TNF-α in whole brain ELISA assessments. TNF-α infusions into the central amygdala (CEA) increased ethanol-induced withdrawal anxiety, as was assessed by a social interaction test. Separately, a CRF1 antagonist administered peripherally prior to TNF-α or MCP-1 ICV infusions reduced ethanol-induced withdrawal anxiety. Together, these two infusion experiments suggest that CRF actions, and not HPA activation, might be underlying these ethanol withdrawal effects. Other studies have supported the role of the HPA axis influencing alcohol consumption (Vendruscolo et al., 2012), but ultimately concluded that CRF activity was more critical for ethanol consumption (Lowery et al., 2010; Koob, 2010). A later study found that a chronic ethanol diet generated increases in high-mobility group box 1 (HMGB1) mRNA 24 hours after cessation from ethanol, as compared to rodents exposed to a control diet. HMGB1 has been previously implicated as mediating stress-induced cytokines, and so this research group hypothesizes that this expression might be due to ethanol-induced

withdrawal effects. In a separate experiment within the same study, this group discovered that an HMGB1 or CRF1 antagonist will reduced ethanol-induced expression of the cytokines (MCP-1, IL-1β, and TNF-α) and HMGB1 24 hours after the last ethanol administration (Whitman et al., 2013). A good review article by Breese and Knapp (2016) describes the interactions between stress, anxiety, CRF, and neuroimmune signaling. Several examples were cited referring to stress leading to relapse drinking, stress leading to greater alcohol drinking, and the frequent co-occurrence of alcohol abuse with psychiatric/stress disorders. Also, see Becker and colleagues (2011) for a review of stress effects on ethanol consumption. Karlsson and colleagues (2016) used to a double knockout mouse which had deletions of IL-1R1 and TNF-1R gene to investigate stress induced drinking due to a social defeat stressor. In their model, double KO mice did not exhibit the stress-induced increases in the ethanol consumption found in control mice.

With the assays used for my dissertation research, the brains were retrieved immediately after the "drinking in the dark" paradigm, and so the IL-6 effects are not likely to be due to acute withdrawal stress effects but IL-6 might be impacted by the neuroadaptations caused by the stress and anxiety found by a history of binge ethanol and withdrawal episodes.

In the Allostasis model, binge cycles of alcohol use and withdrawal alter the normal set-points into an allostatic state. While in the allostatic cycle, prior set-points are no longer attainable. Thus, the perpetual cycles eventually descend into a pathological state (Koob & Le Moal, 2001; Koob, 2003; Koob, 2008). These cycles are easily represented by multiple binge-like cycles of DID in mice. Mice are expected to demonstrate similar behaviors to humans in response to this extended binge-like alcohol exposure. In fact, some studies from the Thiele & Lysle laboratories have supported a potential role that cytokines could be playing over the course of several binge alcohol cycles. For example, Marshall and colleagues (2015) demonstrated that

IL-1ß will show alcohol-induced expression after one binge cycle in the central amygdala and the basolateral amygdala, but will only show this enhanced expression in the basolateral amygdala after three cycles. Marshall and colleagues (2016) discovered that the anti-inflammatory cytokine IL-10 is only increased after three binge cycles, and only in the basolateral amygdala. Adding to these published results, this dissertation work establishes that IL-6 will show alcohol-induced expression after three binge cycles, but only in the PVN and the central amygdala.

In the Allostasis model, the reward circuitry involved with alcohol reinforcement includes the extended amygdala (including the BNST), the lateral hypothalamus, the nucleus accumbens, and the ventral tegmentum area (VTA) (Koob, 2003). These same brain areas (with the exception of the VTA) have been found to host interactions between the neurotransmitters (serotonin and dopamine) implicated in the alcohol Allostasis model and the cytokines IL-1(β), IL-6, and TNF-α (Brebner, Hayley, Zacharko, Merali, & Anisman, 2000). Additionally, the central amygdala was found to host interactions between TNF-α and GABA (Knapp et al, 2011). While IL-6 expression was not increased in the other aforementioned rewards regions within this dissertation, IL-6 was increased in the central amygdala. Notably, the central amygdala has also been a region implicated in the moderation of negative affect, stress, and anxiety behaviors. Recently, Pleil and colleagues (2015) discovered that mice that had been exposed to four cycles of chronic intermittent ethanol and then were tested 48 hours later showed increased anxiety as determined by a marble burying task. Associate with the increased anxiety was an inhibition of signaling in the central amygdala indicative of a reduced capacity for action potential firing.

The innate immune response to pathogens has been found to have interactions with the opioids, glucocorticoids, NPY, and CRF throughout the central nervous system (Sternberg,

2006). Thus, it could be argued that cytokines (such as the homeostatic IL-6) interact with key neurotransmitters and neuropeptides in regions implicated in alcohol reinforcement processes. In fact, IL-6 could be interacting with GABA neurotransmission and affecting the rewarding or aversive properties of binge-like ethanol consumption. The central amygdala is primarily GABAergic, and is strongly implicated in both anxiety and alcohol use disorders (Gilpin et al., 2015). Indeed GABA could be altering CRF expression independently following ethanol exposure (as suggested in Herman et al., 2016), but it is likely that cytokines also contribute to this process. In a series of studies using IL-1 KO mice, IL-1 receptor antagonist, and recombinant IL-1ß, Bajo and colleagues (2014, 2015) established a role for IL-1 in the modulation of ethanol-induced GABAergic neurotransmission in the central amygdala. Interleukin-6 has been implicated to interact with GABA, but studies establishing the ethanol contribution to this interaction have not yet been reported (Garcia-Oscos et al., 2012; Gruol, 2015; Hernandez et al., 2016). While the framework for the allostatic model is not complete, given the cited evidence, it seems likely that cytokines contribute vitally to alcohol dependence/pathology.

Future Directions

Upcoming studies can further characterize the potential roles of the cytokines at the various stages of binge-like ethanol consumption and ethanol-mediated behaviors in the descent into dependence. Also, IL-6 specifically might be tested for potential stress or anxiety roles it might be utilizing to mediate ethanol consumption. In pursuit of this aim, interactions between IL-6 and CRF might also be explored to further establish the interfaces between cytokines and neuropeptides. Alternatively, interactions between IL-6 and GABA could be explored for

potential roles that this interface might have in modulating the rewarding or aversive aspects of ethanol consumption.

New tools are on the horizon for imaging the cytokines with better sensitivity, as well as greater temporal and spatial resolution than existing immunoassays like immunohistochemistry. Bead assays with optical fiber-based sensors, and microfluidic devices have been suggested to be critical tools towards the goal of in vivo detection of the various cytokines acting in response to immune challenges (Liu et al., 2016). Other emerging imaging tools for characterizing immune signaling include nanoparticles, Raman spectroscopy, RNA probes, and MALDI imaging (Jacobsen et al., 2016). Together, some of these tools may inform future studies on cytokine dynamics depending on their practical and effective utility in characterizing immune challenges, such as binge alcohol drinking.

Future studies using IL-6R antagonism might use control antibodies for infusions as opposed to the (vehicle) saline infusions used within these novel studies, but given the specificity of these effects, alternative controls are not expected to alter these particular findings. Indeed, both types of controls have been used previously in the literature (Xin et al., 2014; Narkbunnam et al., 2013), and a thorough search of the Pubmed article database revealed no demonstrated effects with control antibody infusions. Also, IL-6 might also be antagonized through other methods as technology improves. For example: antisense oligonucleotides, mRNA, siRNA, and microRNA gene therapies might prove useful for the antagonism of the various elements of the IL-6 pathway to determine which critical IL-6 gene(s) might be impacting binge-like ethanol drinking. However, these strategies are somewhat limited by the current strategies for delivering these exogenous agents to the critical brain regions for modulating the binge ethanol drinking. Indeed, the challenges, the current progress, and the future directions of these genetic therapies

are explored in greater detail by Yin and colleagues (2014). These genetic strategies may in fact prove to be effective strategies for antagonizing IL-6 with minimal or possibly no cannulation of experimental animals in the near future.

Finally, while there has been a growing focus on the behavioral effects of cytokine actions within the brain, there is much less focus on what neuronal mechanisms that cytokines like IL-6 might be impacting downstream from the IL-6 receptor activation. This presents another potential avenue of exploration for future studies. To review what is known about IL-6 actions, IL-6 may be produced by neurons, microglia, astrocytes, or endothelial cells. IL-6 plays a variety of roles in the body such as regulating inflammation, contributing to neurogenesis, as well as endocrine roles. (Erta, Quintana, & Hidalgo, 2012; Hunter, & Jones, 2015). IL-6 is a critical cytokine that controls the transition from innate to adaptive immune processing. However, the roles of IL-6 may be heavily context dependent (Erta, Quintana, & Hidalgo, 2012). IL-6's role may depend on the level of inflammation in the affected regions. IL-6, in concert with sIL-6Rα has been found to modulate the transition between acute and chronic inflammation.

The two most relevant pathways that IL-6 acts on within the neuroimmune system are the classic signaling and the trans-signaling pathways. Classical signaling is when IL-6 binds to IL-6R and gp130. Classical signaling helps maintain homeostasis and can be neuroprotective. Trans-signaling is when IL-6 binds with sIL-6R and gp130. Trans-signaling is largely responsible for chronic inflammation (and depression) since the pervasiveness of these binding elements allow IL-6 to affect cells that do not express IL-6R. (Erta, Quintana, & Hidalgo, 2012).

In neurons, IL-6 can act as a neuromodulator. IL-6 has been detected in GABAergic neurons, and IL-6R has also been found to express within CRF neurons (Juttler, Tarabin, &

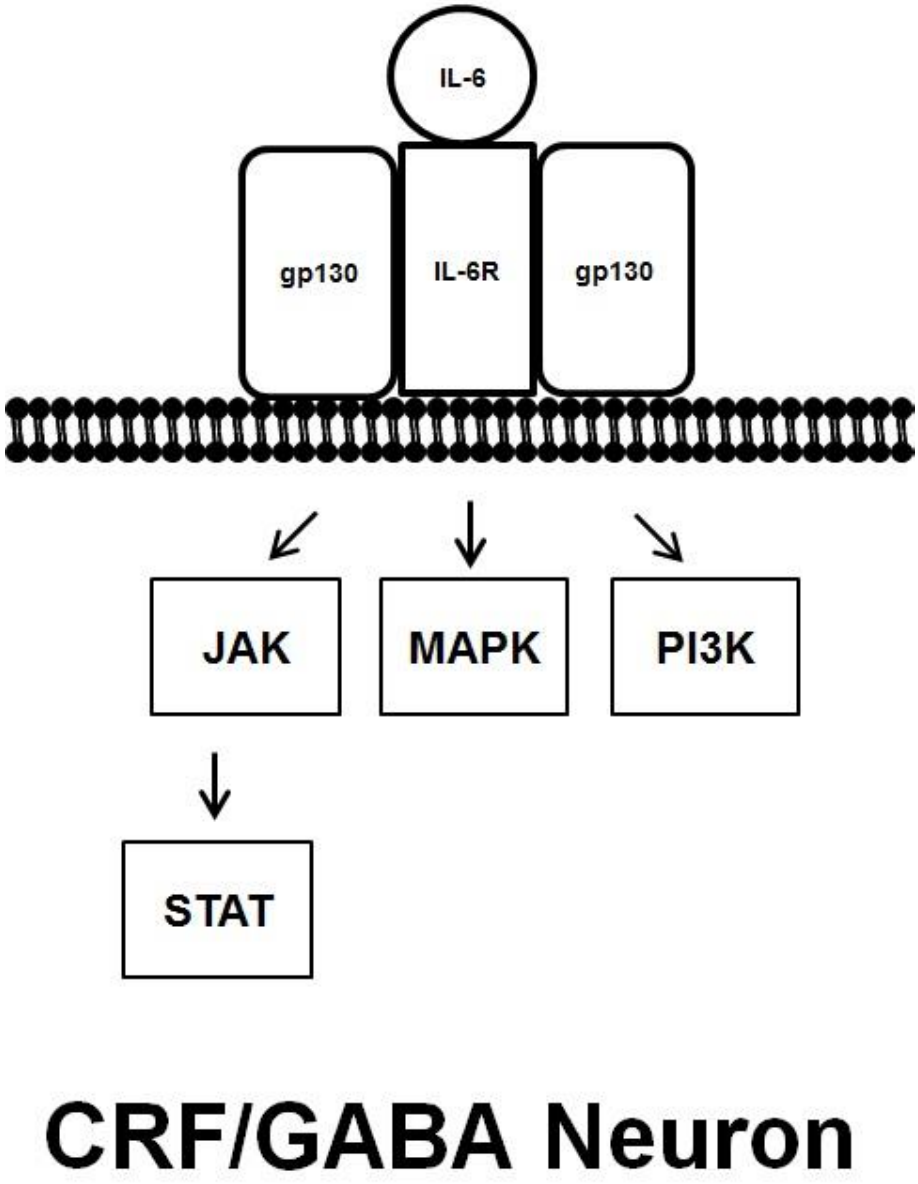
Schwaninger, 2002; Benrick et al., 2009). IL-6 is present in some CRF neurons, but many CRF neurons don't express IL-6R which suggests that trans-signaling IL-6 mechanisms might be responsible for widespread IL-6 expression in CRF neurons. Furthermore, IL-6 is critical in prolonged sickness and infections due to its role in sustaining an extended activation of CRF neurons in the modulation of the HPA axis (Vallieres, & Rivest, 1999). However, IL-6 is considered a neuropoietic cytokine because it promotes neuronal survival. Additionally, IL-6 has been implicated in assisting long-term potentiation, improving spatial learning, as well as maintaining and modulating neurogenesis (Bowen, et al., 2011; del Rey et al., 2013). Depending on the concentrations of IL-6, this cytokine can protect against NMDA excitotoxicity, which is a factor in chronic alcohol drinking. (Juttler, Tarabin, & Schwaninger, 2002).

The central amygdala is primarily GABAergic, and is strongly implicated in both anxiety and alcohol use disorders (Gilpin et al., 2015). Indeed GABA could be altering CRF expression independently following ethanol exposure (as suggested in Herman et al., 2016), but it is likely that cytokines also contribute to this process. Also, Partridge and colleagues (2016) determined through a series of stress related experiments that GABA is the principal co-transmitter in CRF neurons within the central amygdala. In a series of studies using IL-1 KO mice, IL-1 receptor antagonist, and recombinant IL-1ß, Bajo and colleagues (2014, 2015) established a role for IL-1 in the modulation of ethanol-induced GABAergic neurotransmission in the central amygdala. Interleukin-6 has been implicated to interact with GABA, but studies establishing the ethanol contribution to this interaction have not yet been reported (Garcia-Oscos et al., 2012; Gruol, 2015; Hernandez et al., 2016).

While there are still many unknown elements of the IL-6 actions within the neurons, there has been some progress with the intracellular mechanisms. IL-6R receptor activation at the

membrane produces pronounced transcription of the STAT3 and minor production of the STAT1 proteins in the cell, which suggests an IL-6 impact on the JAK/STAT pathway since the JAK2 protein is necessary to activate the STAT3 pathway. Additionally, IL-6 produced phosphorylation of p42/44 MAPK, which suggests an IL-6 impact on the MAPK pathway (Gruol, 2015; Garcia-Oscos et al., 2015; Schumann et al., 1999). Fang and colleagues (2013) used PI3K protein inhibitors to demonstrate that IL-6 also signals through the PI3K pathway. (See **Figure 5.1** for a schematic of IL-6 neuronal actions) Garcia-Oscos and colleagues (2015) suggest a complex role for IL-6 in its interactions with GABAergic signaling that is dependent on the levels and types of IL-6 activations. Trans-signaling is believed to be the dominant form of signaling within the neuron due to the soluble IL-6R's ability to impact many neurons that do not contain, or minimally express membrane bound IL-6R. Each of these pathways has been implicated with cell health, inflammation, and synaptic plasticity memory mechanisms. Indeed, future studies could explore how a history of binge-like ethanol consumption may impact the IL-6 neuronal mechanisms that are believed to be underlying the CRF/GABA neuronal interactions within the central amygdala.

Figure 5.1 A schematic of Interleukin-6 neuronal actions. Converging evidence suggests that IL-6 actions within CRF/GABA neurons of the central amygdala may be underlying the binge-like ethanol drinking effects found within this dissertation. This schematic shows the known JAK/STAT, MAPK, and PI3K pathways that IL-6 impacts within neurons.



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